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Contents

Search Rate and Functional Response of a Eusocial Insect (*Oecophylla longinoda*) in a Tanzanian Mango Orchard

Thora Samsø Fast, Jørgen Aagaard Axelsen, Gina Kier Lynegaard, Maulid Mwatawala, and Joachim Offenberg
Volume 2015, Article ID 817251, 7 pages

Busy Bees: Variation in Insect Flower-Visiting Rates across Multiple Plant Species

Margaret J. Couvillon, Chandra M. Walter, Eluned M. Blows, Tomer J. Czaczkes, Karin L. Alton, and Francis L. W. Ratnieks
Volume 2015, Article ID 134630, 7 pages

The Ant *Cardiocondyla elegans* as Host of the Enigmatic Endoparasitic Fungus *Myrmicinosporidium durum*

Julia Giehr, Jürgen Heinze, and Alexandra Schrempf
Volume 2015, Article ID 364967, 4 pages

An Insight into Sweet Potato Weevils Management: A Review

Seow-Mun Hue and Min-Yang Low
Volume 2015, Article ID 849560, 11 pages

Taxonomy and DNA Sequencing of *Crematogaster coriaria* Mayr, 1872 (Hymenoptera: Formicidae), with Redescriptions of the Worker, Queen, and Male Castes

Shingo Hosoishi and Kazuo Ogata
Volume 2015, Article ID 541351, 8 pages

Body Size of the Monomorphic Ant *Lasius niger*: Young Colonies along a Metal Pollution Gradient

Irena M. Grześ, Mateusz Okrutniak, and Gracjan Antosik
Volume 2015, Article ID 873415, 5 pages

Effects of Pyriproxyfen on Life Table Indices of *Plutella xylostella* in Multigenerations

Mohammad Mahmoudvand, Saeid Moharramipour, and Mehrdad Iranshahi
Volume 2015, Article ID 453701, 7 pages

Behavioral Response of Plum Curculio, *Conotrachelus nenuphar*, to Synthetic Fruit Volatile Lures and the Male-Produced Aggregation Pheromone

Clement Akotsen-Mensah and Henry Y. Fadamiro
Volume 2015, Article ID 350675, 9 pages

Morphology of the Preimaginal Stages of *Lasioptera donacis* Coutin (Diptera: Cecidomyiidae), a Candidate Biocontrol Agent for Giant Arundo Cane

Donald B. Thomas and John A. Goolsby
Volume 2015, Article ID 262678, 11 pages

Diversity and Distribution of Aquatic Insects in Streams of the Mae Klong Watershed, Western Thailand

Witwisitpong Maneechan and Taeng On Prommi
Volume 2015, Article ID 912451, 7 pages

Broad Protein Spectrum in Stored Pollen of Three Stingless Bees from the Chaco Dry Forest in South America (Hymenoptera, Apidae, Meliponini) and Its Ecological Implications

Favio Gerardo Vossler

Volume 2015, Article ID 659538, 7 pages

Observations on the Biology and Anatomy of Myerslopiidae (Hemiptera, Membracoidea)

Roman Rakitov

Volume 2015, Article ID 898063, 10 pages

Dipterofauna Associated with *Sus scrofa* Linné, 1758, Carcasses in Urban and Coastal Regions of São Paulo State, Brazil

Maria Luiza Cavallari, Fabio Navarro Baltazar, Silvio Shigueo Nihei, Daniel Romero Muñoz, and José Eduardo Tolezano

Volume 2015, Article ID 983980, 12 pages

Morphometric Analysis of the Host Effect on Phenotypical Variation of *Belminus ferroae* (Hemiptera: Triatominae)

Claudia Magaly Sandoval Ramirez, Elsa Evelia Nieves Blanco, Reinaldo Gutiérrez Marin, Diego Alexander Jaimes Mendez, Nelcy Ortiz Rodríguez, Fernando Otálora-Luna, and Elis José Aldana

Volume 2015, Article ID 613614, 12 pages

Temporal and Spatial Foraging Behavior of the Larvae of the Fall Webworm *Hyphantria cunea*

Terrence D. Fitzgerald

Volume 2015, Article ID 359765, 6 pages

***Pimachrysa* (Neuroptera: Chrysopidae: Nothochrysinae): Larval Description and Support for Generic Relationships**

Catherine A. Tauber and David K. Faulkner

Volume 2015, Article ID 875738, 19 pages

***Neochrysocharis okazakii* (Hymenoptera: Eulophidae) as a Major Parasitoid Wasp of Stone Leek Leaf Miner *Liriomyza chinensis* (Diptera: Agromyzidae) in Central Vietnam**

Takatoshi Ueno and Dang Hoa Tran

Volume 2015, Article ID 179560, 6 pages

Life History of an Endangered Marine Insect *Halovelvia septentrionalis* Esaki (Hemiptera: Veliidae)

Terumi Ikawa and Aika Nakazawa

Volume 2015, Article ID 712093, 6 pages

Research Article

Search Rate and Functional Response of a Eusocial Insect (*Oecophylla longinoda*) in a Tanzanian Mango Orchard

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Weaver ants, *Oecophylla* spp., are famous for being efficient biological control agents as they prey on a variety of insects, and they are capable of suppressing a large number of pest species. Here, the search rate and functional response of *Oecophylla longinoda* were investigated in a Tanzanian mango orchard using feeding experiments. This was done by following the removal of prey, which constituted the foundation for estimating the search rate by aid of the Nicholson-Bailey and Lotka-Volterra models. The overall mean search rate was 3.2×10^{-4} and 1.7×10^{-4} over 30 minutes, when calculating the search rate using the Nicholson-Bailey equation and the Lotka-Volterra equation (modified Holling equation), respectively. The functional response investigations showed a linear relationship between removed prey and available prey, suggesting type I functional response or, alternatively, the initial phase of type II functional response. The results presented here are probably the first attempt to identify the functional response type of a colony of living predatory eusocial insects.

1. Introduction

Biocontrol using weaver ants, *Oecophylla* spp., has successfully been carried out throughout Asia, Australia [1, 2], and, to some extent, Africa [3, 4]. *Oecophylla* spp. live in colonies in trees where a colony may consist of more than 100 nests and cover many trees, and the two known species have been shown to be beneficial for farmers in multiple ways. Firstly, the ants are generalist predators and control several pest species in a variety of crops such as mango, cashew, coconut, coffee, and mahogany [5, 6]. Using ants as a biological control agent has been shown to increase fruit yield in several crops and may also reduce pesticide costs. For instance, using ants while applying soft chemicals as an integrated pest management strategy in a mango orchard in Darwin, Australia, saved AUD 0.25/tree per year and increased average profit by 73% in comparison to using chemical pesticides [7]. Secondly, these ants are edible and can be harvested and may serve as a protein source for humans or livestock [8, 9]. Thirdly, the anal spots deposited by the ants contain nutrients, which

may be absorbed by the plants and potentially contribute to plant growth [10]. Thus, weaver ants have often proved to be effective biocontrol agents and may furthermore serve as an alternative source of food and fertilizer.

Several factors influence the efficiency of ants as biocontrol agents. Among these are ant densities [11, 12], size and age of colony [13], crop species [6, 14], and climatic conditions [15]. Furthermore, pest dynamics can be affected by predator-prey interactions, involving, for instance, the predator's ability to search for and kill prey. The search rate is a measure of the rate at which the predator encounters prey [16] and for ants may depend on sugar availability [17]. The search rate is an important element in predator-prey interactions and has been included in many mathematical descriptions of predator-prey dynamics such as Holling's disc equation, the simple Lotka-Volterra equations, the Nicholson-Bailey equation, and the Gutierrez-Baumgärtner equation. The latter equation has often been used in simulation models of predator-prey dynamics, as it is built on a representative and

realistic biological basis and because independent field data on predator-prey dynamics can be simulated when applying the equation in simulation models [16, 18, 19]. However, it does not seem to be readily applicable to eusocial insect colonies in cases where it is not possible to estimate the demand for reserves, which is an obligatory element in estimating the total colony demand [16]. Therefore, in this study we used both the Lotka-Volterra equation (produces type I functional response) and the Nicholson-Bailey equation (produces type II functional response) to calculate the search rate, as it is not clear which functional response type is most suitable to describe the predatory behaviour of an ant colony.

The functional response describes foraging capacity and is defined as the amount of the prey consumed as a function of prey density during a certain period of time. There are basically three kinds of functional response types each having a characteristic curve. Type I functional response curve increases linearly where the predator has an increasing rate of prey intake and is produced by the simple Lotka-Volterra equation. This type of functional response is, for instance, representative for spiders waiting passively in their webs until prey is caught [20]. Type II functional response curve increases similarly to type I response curve but decelerates into an upper asymptote at high prey densities as the predator reaches satiation. Most insects follow type II functional response [21] and this type is generally the most common one [22]. The Nicholson-Bailey equation produces type II response. Type III functional response curve has a sigmoid shape and encompasses density dependence where prey-switching occurs at low prey densities. At low prey densities, learning may also occur, which is the ability to achieve a faster handling time with increasing prey density. Type III functional responses are relatively rare for arthropods but have been shown for certain flies, beetles, hemipterans, and lower crustaceans [23–25]. Jeschke et al. [22] have reviewed 48 different functional response equations and by far most of them include handling time and/or predator satiation, and a few of them are even of a dome shaped type IV or have other shapes.

Search rates and functional responses have mainly been investigated for solitary insects of which the foraging activity reflects the demand for prey of an individual. Eusocial insects, on the other hand, forage to fulfil the entire colony's demand for prey, and the colony reproduces as a single unit [26]. In this study, field data were used to investigate (i) the search rate of *O. longinoda* workers in mango trees and (ii) the functional response of *O. longinoda* colonies under natural conditions.

2. Materials and Methods

2.1. The Field Site. The study was conducted in January 2012 on 45, 6-year-old mango (*Mangifera indica*, variety: apple) trees. The spacing between trees was 6×6 meters and the trees were 2.5–3.0 meters high. The study was carried out in the Kibaha district, Coast Region (Pwani), Tanzania (6°44'3"S, 38°43'53"E). The entire study was conducted during the short rainy season with daily temperatures (7 AM–7 PM) ranging between 26.8 and 30.3°C and an average temperature of 29.0°C (weather data from Dar Es Salaam Airport).

2.2. Establishment of Weaver Ant Colonies. *O. longinoda* colonies were transplanted to the experimental trees one month before the study was initiated. Colonies were collected from a nearby citrus orchard by cutting down the available nests by aid of a clipping pole. The nests were rapidly put into plastic bags and brought to the experimental orchard where they were placed in the crown of an experimental tree and torn open to let the ants colonize the tree. All nests from a colony in the citrus plantation were placed in the same experimental tree. The queen's nest was carefully identified from each colony in the citrus plantation and transferred with the other nests from the colony to secure a normal functioning of the transplanted colonies. The transplanted colonies were allowed to colonize six to twelve trees depending on the size of the transplanted colony (number of nests) and the trees were connected by ropes (4 mm in diameter) to facilitate the "traffic" between trees of a colony. After transplantation the colonies were fed with about 10 mL 20% sugar solution (in test tubes attached to the tree) and about 10 g. cat food (placed on the upper side of the main branches) once to boost colony establishment and growth. The trees were pruned to avoid contact between neighbouring colonies.

2.3. Worker Numbers and Densities. Worker ant densities were assessed prior to each experiment by aid of the Forager Density Index (FDI) [9] described in

$$FDI = \frac{((1/3)L + (2/3)Me + H)}{T}, \quad (1)$$

where L , Me , and H were the number of main branches having low, medium, and high densities of ants and T was the total number of main branches. Main branches with low, medium, or high densities of ants were defined as branches having 1–9 ants, 10–50 ants, or >50 ants, respectively. Branches without ants did not count in the index but were included in T .

A relatively high ant activity was required to ensure that experiments reflected situations with good biocontrol, and therefore only trees with $FDI \geq 0.5$ were used in the experiments.

Nest width and length were accurately measured for all nests in all trees using a folding ruler, and the measurements were used to estimate ant numbers based on the equations from Lim [27].

2.4. Field Experiments. Nine ant colonies distributed on 13 trees in total were used to investigate the search rate. Five pieces of 1.5 × 5.0 cm double-sided adhesive tape were pasted haphazardly on the branches (up to two meters height) of each of the nine trees used in the experiments. Tape pieces were never pasted onto branches that had a diameter less than 3 cm. Each piece of tape had 10 equally sized insect prey items, which were evenly distributed, pasted onto it (Figure 1). Prey types included individuals from the orders Lepidoptera, Hemiptera, and Diptera. Prey items were not replaced when removed by the ants, which means the prey density declined with time.

TABLE 1: Average dry-weight (mg) of individual prey items used in experiment.

Order	Diptera (Culicidae)	Lepidoptera	Hemiptera	Diptera (<i>Musca domestica</i>)	Mixed prey types
Average dry-weight (mg)	0.10	0.89	7.62	5.06	4.52

FIGURE 1: *Oecophylla longinoda* removing prey from tape pieces.

In order to estimate the biomass of the prey provided in the experiments, samples of at least 18 individuals of each prey type were collected in the field, dried for 24 hrs at 60°C, and weighed, and the average biomass was calculated. In cases with pieces of tape comprising a mix of prey types, an average biomass was estimated from the average biomasses of the used prey types (Lepidoptera, Hemiptera, and house flies (*Musca domestica*)) (Table 1).

In an experiment five pieces of tape (each with 10 prey items) were pasted in each tree, and every 30 minutes the number of preys left on the tapes was counted. When all prey had either disappeared or 150 minutes had passed, the ongoing experiment was ended. The search rate was calculated for each time interval.

As other predators or wind might also cause prey removal, background disappearance rate was measured in 5 control trees in which ants were not present.

The functional response of *O. longinoda* was investigated for 13 ant colonies distributed on a total of 29 trees. Each tree had the same experimental set-up as used to investigate the search rate; however, prey numbers per tree ranged from 10 to 200, that, is 1–20 pieces of tape with 10 prey items each. The only prey type in this experiment was (*Musca domestica*) having an average dry-weight of 5.06 mg. The experiment was stopped after 30 minutes when the numbers of prey left on the tapes were counted.

2.5. Data Treatment. The search rates were initially estimated using a modified version of Holling's disc equation [28], which reduces to a Lotka-Volterra equation (representing type I) when handling time can be ignored. Ignoring handling time seems acceptable in the case of an ant colony because even when a large group of ants is handling a larger prey, there will normally still be immense numbers of other ants

searching for prey. Ignoring the handling time in Holling's disc equation reduced it to

$$s(\Delta t) = \frac{M^*}{N_w \cdot M'}, \quad (2)$$

where $s(\Delta t)$ is the ants' search rate in the time interval Δt , M^* is the food acquired by the predator measured as the amount that disappears during the experiment, M' is the available biomass of prey, and N_w is the number of workers. Another functional response equation, which represents type II response, is the Nicholson-Bailey equation [29]

$$M^* = M' \times (1 - e^{-s(\Delta t) \times N_w})$$

$$\iff s(\Delta t) = \frac{-\ln(1 - M^*/M')}{N_w}. \quad (3)$$

This equation is independent of both demand rate and handling time and assumes that predators are searching randomly, which means that a group of predators (such as the workers from an ant colony) may search overlapping areas. These two equations, (2) and (3), seem to be good candidates to describe the functional response of a colony of predatory eusocial insects while most other functional response equations reviewed by Jeschke et al. [22] do not seem to be applicable as they have handling time and satiation as key parameters, parameters that are either difficult to estimate at colony level (satiation) or seem to be irrelevant (handling time) due to the large number of searching workers although some individuals are busy handling preys.

The total number of workers in (3) was estimated by correlating to nest size, using the equation by Lim [27]:

$$\ln N_{\text{ants}} = -1.16 + 1.09 \ln V, \quad (4)$$

where V is the volume of a nest calculated by

$$V = 2 \times l \times r \times \pi, \quad (5)$$

where r is the nest radius in cm ($= (1/2)\text{height}$) and l is the length of the nest in cm.

2.6. Statistical Treatment. The search rate was tested for dependence on colony, colony size, tree crown volume, and time interval by aid of one-way analyses of variance (ANOVA). Similarly, concerning the functional response the dependence of the amount of removed prey on the amount of prey available was tested by aid of ANOVA, and the fits of data to functional response types were investigated by comparing R^2 -values of linear and logarithmic trend lines.

TABLE 2: Cumulated percentage prey detection after $t = 0$ and the cumulated background disappearance rate.

Interval (min)	0–30	30–60	60–90	90–120
Percentage of tape pieces discovered after $t = 0$	77	91	100	100
Background disappearance rate (%)	0	1.76	1.76	2.35

3. Results

3.1. Field Observations: Background Disappearance Rate. It was observed that *O. longinoda* removed and carried preys from tape pieces to their nests, ensuring that data reflects ant activity and that preys were not removed by, for example, wind or other animals. In all experiments, ants detected prey on at least one piece of tape within the first time interval. Background disappearance rate ranged between 0 and 2.35% of the mass of prey items depending on the time interval and was subtracted from the removed biomass before data treatment (Table 2).

3.2. Field Observations: Prey Removals. The total amount of prey that disappeared from the tapes (M^*) measured in mg dry-weight per colony during the 30-minute time intervals is summarised in Table 3. Biomass of prey provided (M') at $t = 0$ varied according to prey type; therefore the percentage of prey removal within a given time interval is also summarised. The time interval prey removal rates varied between 0 and 100% and showed no tendency to decrease or increase with time.

3.3. Search Rate. An average search rate for each time interval was calculated for all colonies (Table 4), and the search rate was not significantly dependent on the time interval regardless of which equation was used to calculate the search rate (ANOVA, d.f. = 4, $F = 0.32$, and $P = 0.86$ using Lotka-Volterra and d.f. = 4, $F = 2.26$, and $P = 0.08$ using Nicholson-Bailey). Instead, the search rate was significantly different between colonies when using Lotka-Volterra (ANOVA, d.f. = 8, $F = 6.34$, and $P = 0.001$) but not significantly different between colonies using Nicholson-Bailey (ANOVA, d.f. = 8, $F = 1.70$, and $P = 0.13$ using Nicholson-Bailey). The overall mean search rate was 3.2×10^{-4} and 1.7×10^{-4} over 30 minutes when calculating the search rate using the Nicholson-Bailey equation and the Lotka-Volterra equation (modified Holling equation), respectively. The search rate comes in units of time^{-1} , and therefore it makes sense to convert the search rate to units of hour^{-1} giving an average search rate of $6.4 \times 10^{-4} \text{ ant}^{-1} \times \text{hour}^{-1}$ and $3.4 \times 10^{-4} \text{ ant}^{-1} \times \text{hour}^{-1}$ using the two equations, respectively. There is a rather large variation in search rates between the colonies, from 0.9×10^{-4} to 10.9×10^{-4} using the Nicholson-Bailey equation and from $0.8 \times 10^{-4} \text{ ant}^{-1} \times \text{hour}^{-1}$ to $5.7 \times 10^{-4} \text{ ant}^{-1} \times \text{hour}^{-1}$ using the Lotka-Volterra equation. There was no significant dependency between search rate and colony size (ANOVA, d.f. = 1, 42, $F = 2.68$, and $P = 0.11$ using Lotka-Volterra and $F = 0.16$, $P = 0.69$ using Nicholson-Bailey) or between

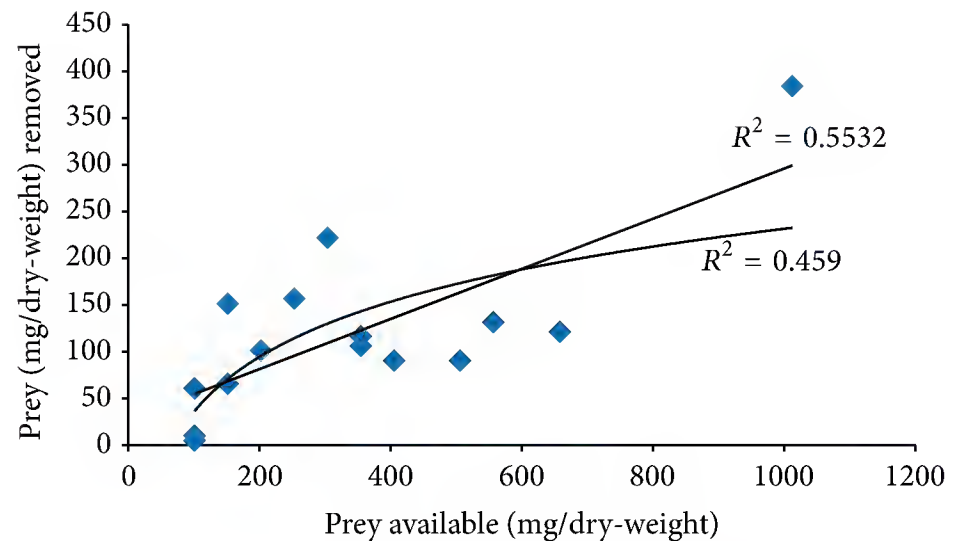


FIGURE 2: Linear and logarithmic trend lines representing type I and type II functional response functions, respectively, compared to observed data.

search rate and tree crown volume (ANOVA, d.f. = 1, 42, $F = 0.94$, and $P = 0.034$ using Lotka-Volterra and $F = 0.27$, $P = 0.61$ using Nicholson-Bailey).

3.4. Functional Response. The amount of prey removed was significantly dependent on the amount of prey available relationship (ANOVA: d.f. = 1, $F = 16.10$, and $P = 0.002$), and a linear trend line showed larger R^2 -value (0.55) than a logarithmic trend line (0.46) (Figure 2). This result indicates type I functional response or, alternatively, the initial part of type II that has not reached satiation. Similar analyses were also carried out expressing the removed prey in units of “prey removed per worker (g prey/worker)” and “prey removed per worker per tree crown volume” (g prey/worker/ m^3), but the R^2 -values were clearly lower (0.24 and 8×10^{-3} , resp.). Furthermore, the significant dependence of the amount of removed prey on the amount of available prey disappeared when using these units (ANOVA: d.f. = 1, 14, $F = 3.75$, and $P = 0.08$ (g prey/worker) and d.f. = 1, 14, $F = 0.10$, and $P = 0.75$ (g prey/worker/ m^3)).

4. Discussion

4.1. Functional Response. When investigating the functional response of *O. longinoda*, a linear relationship revealed the largest R^2 -value (Figure 2), and the relationship was significant when neither ant density nor tree crown volume was included. The trend line on the graph indicated no sign of satiation of *O. longinoda*, and therefore the results indicated type I functional response or initiating type II functional response.

Oecophylla spp. and ants in general are known to be opportunistic feeders and aggressive hunters [30]. Workers forage in randomly or slightly clumped distributions, with more foragers out in a starved colony under which conditions cannibalism of brood also occurs [31, 32]. When large prey is detected, chemical cues are used to recruit workers [33, 34] and ants are known to bring down large prey including vertebrates such as lizards and birds, of which remains have also been found in dissected *Oecophylla* nests (J. Offenberg, personal observation; [34, 35]).

TABLE 3: Total removed prey (M^*) per tree measured in mg dry-weight and the prey removal rate (%) within each time interval. The removal rate (5) was based on the amount available at the start of each interval.

Colony	Time interval (min)				
	$t = 0-30$	$t = 30-60$	$t = 60-90$	$t = 90-120$	$t = 120-150$
1	22.1 (9.8%)	110.5 (55.2%)	57.4 (64.6%)	17.7 (57.1%)	8.8 (66.7%)
2	4.4 (4.9%)	0	8.8 (10.5%)	0	0
3	26.5 (14.6%)	22.1 (14.5%)	13.3 (10.2%)	0	0
4	44.2 (30.6%)	14.1 (14.5%)	13.3 (15.9%)	23.8 (34.2%)	7.0 (15.3%)
5	153.2 (75.7%)	14.8 (33.1%)	19.8 (66.3%)	4.9 (50.0%)	4.9 (100.0%)
6	9.9 (9.8%)	29.6 (33.1%)	4.9 (8.3%)	0	14.8 (27.3%)
7	8.4 (40.3%)	1.4 (11.4%)	0	0	0
8	45.0 (57.4%)	14.9 (47.0%)	15.0 (89.7%)	0.1 (6.3%)	0
9	5.4 (18.6%)	14.0 (61.2%)	2.6 (29.7%)	0.9 (14.2%)	0.9 (16.5%)

TABLE 4: The search rates from the 9 trees calculated according to the Nicholson-Bailey equation (upper line in normal font) and Lotka-Volterra (lower lines in italics). All figures are $\times 10^{-4} \text{ ant}^{-1} \times 30 \text{ minutes}^{-1}$.

Colony	Ant density	Crown volume (m^3)	Int. 1	Int. 2	Int. 3	Int. 4	Int. 5	Avg.	S.E.
1	1216	4.9	0.9	19.2	7.8	1.7	0.0	5.9	6.7
			<i>0.8</i>	<i>4.8</i>	<i>5.0</i>	<i>3.7</i>	<i>0.41</i>	3.7	1.8
2	346	3.1	1.5	0.0	3.2	0.0	0.0	0.9	1.2
			<i>1.4</i>	<i>0.0</i>	<i>2.9</i>	<i>0.0</i>	<i>0.0</i>	0.9	1.1
3	687	2.1	2.0	2.6	1.5	0.0	0.0	1.2	1.7
			<i>1.7</i>	<i>2.1</i>	<i>1.3</i>	<i>0.0</i>	<i>0.0</i>	1.0	0.6
4	2436	3.6	1.4	1.0	1.0	1.9	0.8	1.2	1.7
			<i>1.0</i>	<i>0.7</i>	<i>0.8</i>	<i>1.2</i>	<i>1.5</i>	1.0	0.6
5	1123	1.4	17.1	2.7	20.5	0.0		10.1	12.9
			<i>6.7</i>	<i>1.8</i>	<i>6.4</i>	<i>4.5</i>	<i>8.9</i>	5.7	2.1
6	1611	2.4	0.7	4.2	2.1	0.0	1.4	1.7	1.8
			<i>0.6</i>	<i>2.3</i>	<i>1.5</i>	<i>0.0</i>	<i>3.7</i>	1.6	1.1
7	1240	3.6	3.7	2.3	0.0	0.0	0.0	1.2	1.4
			<i>2.4</i>	<i>1.8</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	0.8	0.3
8	873	4.0	4.6	2.6	20.0	0.9	0.0	5.6	5.1
			<i>3.4</i>	<i>1.9</i>	<i>4.3</i>	<i>0.8</i>	<i>0.0</i>	2.1	1.6
9	3014	6.6	1.8	7.5	1.0	0.3	0.8	2.3	3.0
			<i>1.0</i>	<i>1.8</i>	<i>0.7</i>	<i>0.3</i>	<i>0.6</i>	0.9	0.5
Avg.	1394	3.5	3.8	5.5	5.7	0.5	0.3	3.2	
			2.1	2.1	2.3	1.0	1.2	1.7	
S.E.	253	0.5	1.1	1.8	2.4	0.3	0.2	1.1	
			2.5	2.6	3.0	2.3	2.6	2.6	

Colony maintenance and growth depend on several factors. The brood needs protein for growth and the queen for egg production; workers mainly need sugar for activity and respiration [36]. A surplus of protein may be invested in reproduction of sexuals, thus increasing the fitness of the colony [37]. Only a fraction of the colony is known to forage, and the distribution of prey and other resources within the colony is therefore essential [15, 38]. When resources are scarce or environmental impacts are unpredictable there is a need for storing existing resources [39]. A study by Rastogi [33] revealed that ants cover large prey with leaves and twigs for hiding. Storage can also be obtained by workers laying trophic eggs [35, 39, 40] and, in addition, ants are suspected of

spraying formic acid on their prey for preservation (J. Offenberg, personal comment). Conservation of prey is an evolutionarily sound approach, especially in the time before the rainy season in which larval production is increased [15]. In small colonies where environmental stochasticity may have a major effect on the survival of a colony, storage of resources may thus have a major effect on colony persistence.

Displaying type I functional response, which does not describe any kind of saturation, may therefore create conditions under which more food than demanded may be acquired, hence improving the chance of survival if a food shortage occurs. This is an ecologically advantageous behaviour and it fits well with the observations made, and

it suggests using the Lotka-Volterra equation to calculate the functional response in calculating predator-prey relationships. On the other hand, if what we see in our investigation of the functional response is in reality the start of type II functional response, the Nicholson-Baily equation is the obvious choice. As long as this issue has not been settled, it seems reasonable to calculate search rates using equations producing both type I and type II functional response.

Despite a thorough search in literature databases it was not possible to find papers describing attempts to quantify the functional response of ants for comparison. However, by expanding the field to include other eusocial insects such as honey bees and termites we found one paper. Sih and Baltus [41] investigated the functional response of honey bees (*Apis mellifera*) and bumble bees (*Bombus* spp.) by relating the patch size with catnip (*Nepeta cataria*) and in turn the number of available flowers to the visiting frequency of the bees. Their results suggested that the bumble bees showed type I response ($R^2 = 0.73$) and the results on honey bees weakly suggested type II response ($R^2 = 0.43$). Therefore, the use of type I response to describe prey acquisition by eusocial insects is weakly supported but the evidence is definitely not strong.

Colonies of eusocial insects can be regarded as super [42], and a colony response to increased density of prey at a certain site requires communication within the colony. Eusocial insect colonies are known to have advanced communication systems [42], but there may be a delay between message and response. Satiation of colony demands for sugar and/or prey may take time, and it may also take time until the message that the colony is satiated spreads to all workers. Therefore, investigations of functional response of eusocial insects should probably try to take such a time delay into consideration to get a colony response to increasing resources. Otherwise, type I response will be most likely. In order to demonstrate an accelerating search rate (type III response) the colony should recruit more workers to the resource. This may happen if the resource is dense enough to trigger an aggregated response, and therefore a large resource of relatively scattered preys may cause a weaker response than a resource of the same size concentrated on little space such as dead larger insects or dead vertebrates. The first situation may show type I response, and the second situation may show type III response. Therefore, investigating the functional response of colonies of eusocial insects is much more complicated than investigating the functional response of individual insects.

4.2. Search Rate. The search rate of *O. longinoda* was found to be dependent on the colony and there is about a factor 10 between the smallest and the largest value. There may be a number of possible reasons for variation in search rate, including state of the colony (young, mature, and old), level of satiation for sugar and/or prey, and season of the year. In this study, we have been using newly established colonies only, which may have reduced the range. However, their original age and demands for prey and sugar remained unknown. Therefore, this study which seems to be one of the first attempts to quantify the search rate and functional response

of eusocial insects constitutes a first step, and the results are valid as such. However, in future investigations of the search rate of *O. longinoda* or other eusocial insects it should be attempted to control the factors causing variation, such as state of colony and demand rate for sugar and/or protein.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Busy Bees: Variation in Insect Flower-Visiting Rates across Multiple Plant Species

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We quantified insect visitation rates by counting how many flowers/inflorescences were probed per unit time for five plant species (four native and one garden: California lilac, bramble, ragwort, wild marjoram, and ivy) growing in Sussex, United Kingdom, by following individual insects ($n = 2987$) from nine functional groups (honey bees (*Apis mellifera*), bumble bees (*Bombus* spp.), hoverflies, flies, butterflies, beetles, wasps, non-Apidae bees, and moths). Additionally, we made a census of the insect diversity on the studied plant species. Overall we found that insect groups differed greatly in their rate of flower visits ($P < 2.2e^{-16}$), with bumble bees and honey bees visiting significantly more flowers per time (11.5 and 9.2 flowers/minute, resp.) than the other insect groups. Additionally, we report on a within-group difference in the non-Apidae bees, where the genus *Osmia*, which is often suggested as an alternative to honey bees as a managed pollinator, was very speedy (13.4 flowers/minute) compared to the other non-Apidae bees (4.3 flowers/minute). Our census showed that the plants attracted a range of insects, with the honey bee as the most abundant visitor (34%). Therefore, rate differences cannot be explained by particular specializations. Lastly, we discuss potential implications of our conclusions for pollination.

1. Introduction

Many factors, both plant- and insect-centered, can affect pollination. From the plant perspective, these may include quality, quantity, and temporal/spatial variation of pollen production and the receptivity of the stigma [1–13]. Because insect pollination is a generally indirect effect of foraging behavior (with some well-known exceptions), plants have also evolved adaptations that influence pollinating insects, such as providing rewards, developing nectar guides to help direct pollinator movement, and placing the rewards so that the pollinator must contact the stamens or stigma [14].

In addition, pollinating insects themselves also have various attributes that affect their efficacy as pollinators, such as how much pollen they typically collect on their bodies [15, 16] and their degree of flower constancy [17]. Another important factor contributing to pollination is the movement

of pollinators between flowers, where a higher visitation rate should result in a greater amount of pollination, if all else is equal.

Previously, flower visitation rate has been mostly investigated from the plant perspective, where researchers quantify visits by different pollinators to a particular plant [2, 18–26]. However, any variation in visitation rate among different pollinators on a particular plant species may reflect a specialization or advantage of that particular pollinator-plant pair, such as the proficiency of hummingbirds on larkspur (compared to bumble bees) or bumble bees on lavender (compared to honey bees [19, 27]). What is less known is whether there are general, broadly consistent differences in the visitation rate between insect functional groups. In other words, we wished to know more about flower visitation rates from the perspective of the insect across different flowering plants.

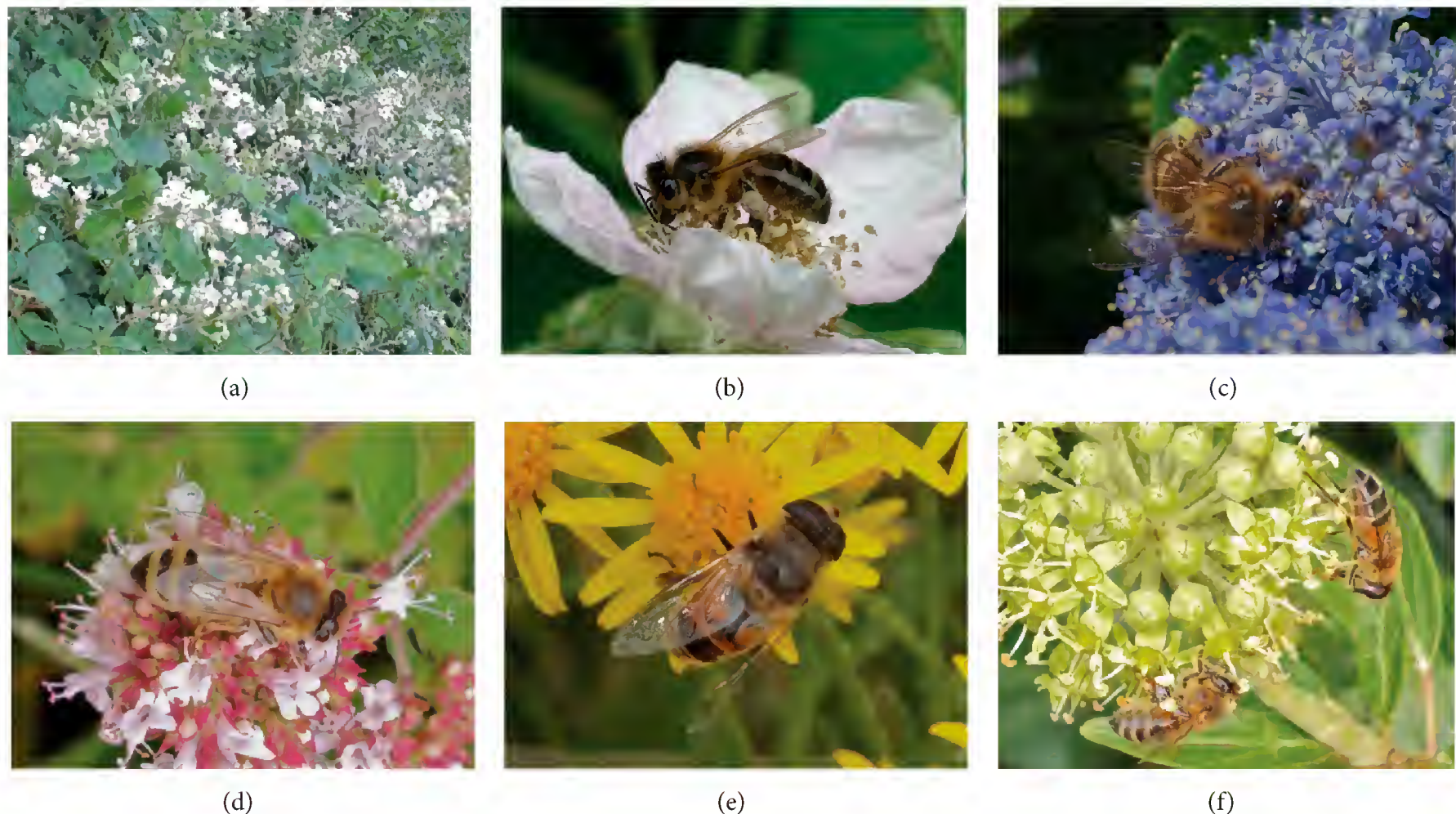


FIGURE 1: We investigated insect visitation rate on five plant study species: bramble (*Rubus fruticosus* (a), (b)), California lilac (*Ceanothus* spp. (c)), marjoram (*Origanum vulgare* (d)), ragwort (*Jacobaea vulgaris* (e)), and ivy (*Hedera helix* and *H. hibernica* spp. (f)). Other than California lilac, a garden shrub, all plants are native to the study area and all five plants were abundant and found in large patches (a). All insects seen are honey bees, with the exception of the similar-sized hoverfly *Eristalis* spp. on ragwort in (e) and on the right on ivy in (f) (insect on the left in (f) is honey bee).

2. Materials and Methods

2.1. Study Locations, Plants, and Insect Identification. Our data were collected at six different locations in southeast England (Albury Downs, Surrey; Ardingly, West Sussex; Herstmonceux, East Sussex; Falmer, East Sussex; Wivelsfield, East Sussex; Magham Down, East Sussex). Our goal was to collect rate and census data of insects foraging on each plant species from a minimum of two locations. Locations growing the same plant may differ in microclimate (e.g., different sunshine or wind exposure or soil type) and other factors (e.g., proximity to a nesting aggregation of a particular insect type), so we retained location as a random factor within the model (see below for details).

We studied insect visitation on five plants (Figure 1): bramble (*Rubus fruticosus*), California lilac (*Ceanothus* spp.), marjoram (*Origanum vulgare*), ragwort (*Jacobaea vulgaris*), and ivy (*Hedera helix* and *H. hibernica*). All except *Ceanothus*, a shrub native to California and commonly grown in UK gardens, were native. We studied a number of plant species because we wanted to know whether there were general differences among insects in visitation rates. We chose plants that were in abundance at different locations, were highly attractive to insects, and were attracting a variety of insect types.

Flower morphology and the arrangement of flowers in inflorescences vary between plants (Figure 1). As we were interested in relative differences between insects across

plants, the manner in which flower visits were counted needed only to be consistent within plant type, irrespective of insect. Therefore, we defined “flower visit” in a way that was practical and ecologically relevant for each plant. For bramble, each flower is large (diameter c. 3 cm), and it is possible to identify insect visits to individual flowers (Figure 1(b)). Because ragwort (Asteraceae) has distinct composite flowers (capitulum, diameter c. 2 cm; Figure 1(e)), we considered a capitulum visit to be the measurement unit. Lastly, California lilac, marjoram, and ivy all possess small flowers (diameter c. few millimeters; Figures 1(c), 1(d), and 1(f)) clustered tightly into inflorescences. For these, we found it impractical to quantify visits to individual flowers and instead defined a flower visit as an inflorescence visit.

Because some of our data were collected by nonexpert insect enthusiasts, we were limited in our taxonomic resolution. Some insects could be identified to species (e.g., honey bees, bumble bees, and butterflies), even in the field as they foraged. However, as we did not collect the insects for later identification, others (hoverflies of the genus *Eristalis*, many of the non-Apidae bees) proved more difficult. Therefore, although we recorded species if it were known, we confined our analysis to nine broad functional groups: honey bees, bumble bees (*Bombus* spp.), butterflies, hoverflies, non-Apidae bees, beetles, flies (nonhoverflies), moths, and wasps. All of the rate data (flowers/minute) were collected on insects that fall within these groups. The census data (see below) used the same groups, with the exception of moths, which

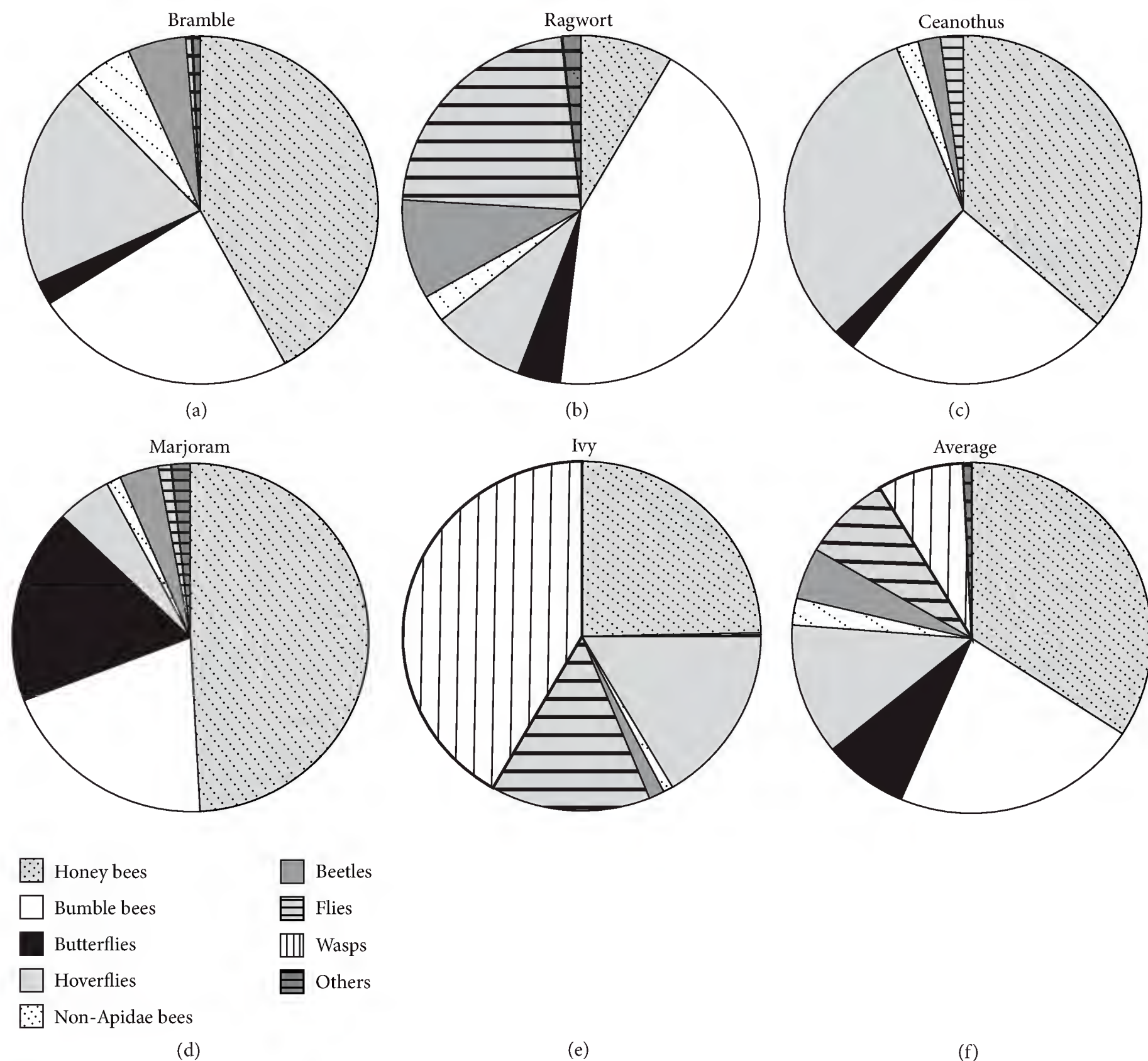


FIGURE 2: Eight main insect groups were commonly found on all plants in varying proportions. Daily census data were taken on (a) bramble, $n = 900$; (b) marjoram, $n = 1300$; (c) ragwort, $n = 800$; and (d) ivy, $n = 700$. California lilac (e) census was inferred from the pilot rate data ($n = 99$). Averages (f) were taken across all the data. The “Others” category, which was infrequent ($<2\%$ of all collected census data), included ants ($n = 6$), mirid bugs ($n = 5$), a lacewing ($n = 1$), and moths ($n = 28$). Overall we took a census on 37 study days across four plants ($n = 3700$ for bramble, marjoram, ragwort, and ivy plus $n = 99$ for California lilac). Honey bees were the most frequent insect observed (34%), followed by the bumble bees (22%) and hoverflies (12%).

were rare enough in the overall census to be combined with others (Figure 2, “Others” category). Census data also additionally included a few ants, bugs (Miridae), and lacewings (Chrysopidae), but these were infrequent, comprising $<2\%$ of the insects counted, and were also included with moths in the “Others” category (Figure 2).

2.2. Data Collection: Insect Visitation Rate. Data were collected from June 27 to October 30, 2013, on days when the insects were actively foraging (12–31°C). The number of days spent collecting data per plant was largely determined by bloom period and days with good weather and was as follows: California lilac: 7; bramble: 18; marjoram: 17; ragwort: 7;

ivy: 10. Once an insect was spotted, we would count the number of “flowers” (see above for definitions) probed in one minute, with the stopwatch starting with the first probing. The plants were found in large, adjacent patches (bramble or ivy) or were many plants growing close together (ragwort, marjoram; Figure 1(a)), which aided in our following of the insects. Once we completed a rate count for one insect, we would walk further down the patch or to another plant to find a different insect, which decreases the likelihood of pseudo-replication. Usually it would take nearly an hour to walk down the patches.

We also noted whether, during the one-minute observation period, the insect left the target plant to visit a different,

neighboring plant species. This was a rare occurrence (12 times over the entire study period, 0.004% of visits), even though all of our target plant locations were in natural or seminatural landscape (with the exception of the California lilac, which was planted on campus) and amid other flowering plants and trees. Lastly, less data were collected on California lilac ($n = 99$), although we still include these data within the main analysis, as plant is retained as a random factor. In all, we gathered one-minute flower visitation rate data for 2987 insects from the five study plants across the entire study.

2.3. Data Collection: Insect Census. To determine which types of insects were generally present, we also carried out a census of the first 100 insects observed on each plant at the start of every study day. Census-taking was less to determine all the insects that visit plants, where it would be necessary to take time of day into account, and more to obtain a broad picture of insect visitors. Census-taking involved walking slowly through or by the entire plant patch and identifying 100 insects on the flowers. We did not collect formal census data on California lilac because we used this species in the beginning as a pilot before we had agreed upon the methods. However, as we collected $n = 99$ rate data points (see above), we used these data to provide a census for California lilac. In all, we counted and categorized 3799 insects (bramble = 900, marjoram = 1300, ragwort = 800, ivy = 700, and California lilac = 99) for the census.

2.4. Data Analysis. For the census data, we report only descriptive statistics as here the main aim was simply to provide a broad picture of the abundance of different insect types per plant species and to show that each plant was visited by a range of insects.

For the differences among insect types in flower visitation rate, which was the main study question, all analyses were done in R 3.0.2 using the lmer function from the lme4 package [28, 29]. We used generalized linear mixed models (GLMMs) with a Poisson error structure. Location and plant species were included as random factors. We tested the response of rate (flower visitation rate) against the fixed factor of insect group (9 categories, see Figure 3/Table 1).

Additionally, during data collection, it was clear that there were large differences within the non-Apidae bees. Specifically, we noticed that Megachilidae bees were quicker compared to the others, mostly Halictidae. Within Megachilidae, we were able to identify *Osmia bicornis* (previously *Osmia rufa*). Therefore, to investigate further within-group differences, we performed an analysis in which we separated out the non-Apidae bees to *Osmia* and non-*Osmia*. Then we used a similar model to test for differences in visitation rate between these two and also compared to honey bees. For both models, we performed necessary corrections for multiple testing [30], and all reported p values are adjusted.

3. Results

3.1. Census of Insect Present: A Variety of Insect Visitors Were Found on All 5 Plant Species. Of our nine insect functional

TABLE 1: Summary of rate data showing number of observations, the mean rate, and the post hoc significance. Rows are ordered according to visitation rate. In all, we collected rate data (flowers probed/minute) from 2987 insects.

Insect group	Observations (n)	Mean rate/minute	Post hoc significance
Bumble bees	524	11.5	a
Honey bees	808	9.2	b
Non-Apidae bees	114	5.2	c
Hoverflies	479	4.8	d
Wasps	128	4.3	e
Butterflies	668	3.9	f
Flies (others)	52	3.4	gf
Moths	81	3.2	g
Beetles	133	2.2	h

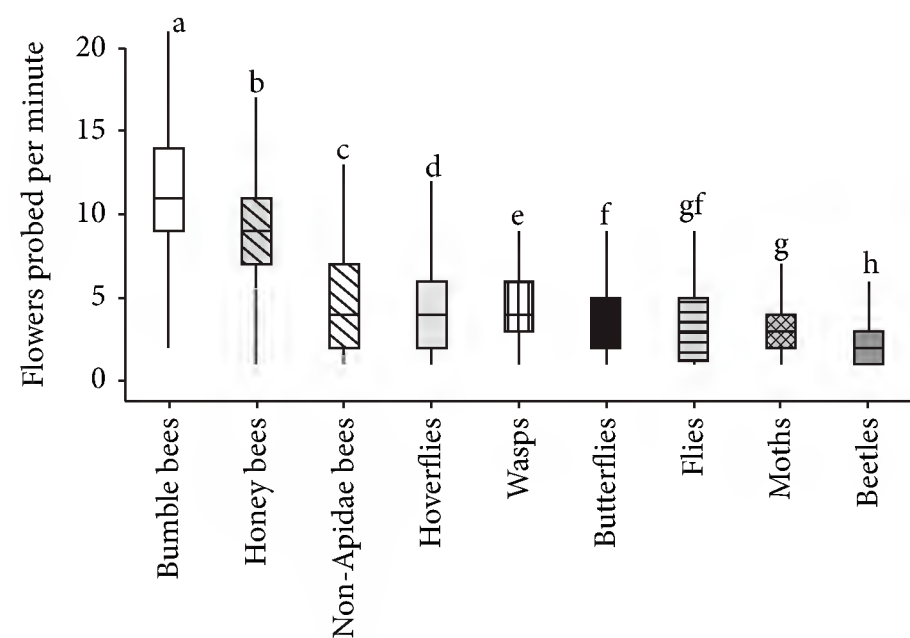


FIGURE 3: Insect pollinator groups significantly differ in the rate at which they probe flowers. Bumble bees (11.5 flowers/minute) and honey bees (9.2 flowers/minute) have the quickest rate of flower visitation, whereas beetles are the slowest (2.2 flowers/minute). Letters display post hoc results, where groups that share letters do not significantly differ. All pairwise comparisons significantly differed from each other, with the exception of flies and butterflies as well as flies and moths. Groups are ranked according to their flower probing rate. Box lines report medians and lower and upper quartiles, and whiskers extend either to maximum or minimum data points or to 1.5 times the interquartile range.

groups, we found eight (honey bees, bumble bees, butterflies, hoverflies, non-Apidae bees, beetles, flies, and wasps) in abundance on all five plants in varying proportions ($n = 3799$, Figure 2). There was some seasonal effect. For example, wasps (most are Vespidae) are most common on ivy, which blooms in autumn, presumably because their colonies reach maximum population at this time of year [31]. In contrast, bumble bees were not abundant on ivy, as their colonies generally die out in late summer [32]. The honey bee was the most abundant insect overall in the census (34%), followed by bumble bees (22%) and hoverflies (12%; Figure 2(f)).

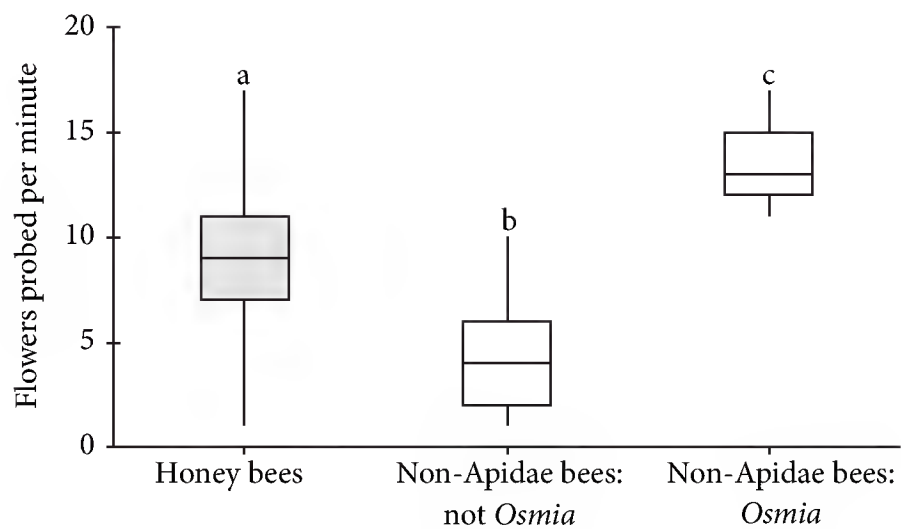


FIGURE 4: *Osmia* spp. visit significantly more flowers (13.4/minute) than other non-*Osmia* solitary bees (4.3/minute) and honey bees (9.2/minute). Letters display post hoc results, where groups that share letters do not significantly differ. Box lines report medians, and whiskers extend either to maximum or minimum data points or to their 1.5 times the interquartile range.

3.2. Insect Groups Differ Significantly in Their Flower Probing Rates, regardless of Plant. There was a highly significant effect of insect group on rate ($n = 2987$; Table 1, Figure 3, GLMM with group as fixed factor compared to the null model GLMM without group, $\chi^2 = 3896.2$, $df = 8$, and $P < 2.2e^{-16}$). The mean rate ranged from bumble bees (11.5 flowers/minute) to beetles (2.2 flowers/minute; Table 1). Post hoc analysis revealed that all pairwise comparisons were significantly different from each other except between butterflies and flies as well as flies and moths (Figure 3). Location and plant explained little of the variance (0.011 and 0.062, resp.), although we retained them as random factors in the model.

3.3. *Osmia* spp. Have a Significantly Faster Flower Visitation Rate Than Other Non-Apidae Bees. When we looked specifically at *Osmia*, we found a highly significant effect of group (*Apis*, non-Apidae: not *Osmia*, and non-Apidae: *Osmia*) on rate (Figure 4, GLMM with group as fixed factor compared to null model without group, $P < 2.2e^{-16}$). *Osmia* spp. had a higher rate (13.4 flowers/minute) than honey bees (9.2 flowers/min) and other non-*Osmia* non-Apidae bees (4.3 flowers/minute).

4. Discussion

Over 500 years ago, Geoffrey Chaucer coined the phrase “as busy as bees” in his *Canterbury Tales*. Centuries on, our results confirm that this widely accepted expression is in fact true regarding bee foraging behavior. Bees, especially the numerically abundant Apidae bees, visited many more flowers per minute than other types of insects. The bumble bees, as the quickest visitors (11.5 flowers/minute), were over five times faster than the beetles, which were the slowest (2.2 flowers/minute; Figure 3, Table 1). This is in agreement with previous work demonstrating that hymenopterans were quicker than lepidopterans or dipterans in their visitation

rates on one plant, lavender, in a study that described interspecific patterns of plant-pollinator interactions [2]. Our results span five different plant species and describe what may be a general phenomenon, although this will need confirmation with additional studies in other countries and climates and on different insects within each category (e.g., stingless bees).

However, not all bees were busy. Within the non-Apidae bees, while *Osmia* spp. had a speedy 13.4 flowers/minute rate, the other non-*Osmia* non-Apidae bees visited 4.3 flowers/minute (Figure 4). Many of the slower non-*Osmia* non-Apidae bees were quite small, mainly of the family Halictidae. Further research could determine if there are consistent differences in visitation rate between the two families. The quickness of *Osmia* is especially interesting because the genus is often suggested as a good wild bee pollinator for orchards. *Osmia* are possible to manage and also have a short flight range, which means they will not stray far when placed in an orchard, and are tolerant of nonideal weather conditions, like wind and light rain [8, 33–35]. Here we show that *Osmia* also possesses a quick flower visitation rate, which has previously been reported for berry pollination [35] and may also help them to be effective pollinators.

In our study, we did not control for temperature, which influences the activity levels of insects. However, our census data shows that most insects were found on most plants, which bloomed successively from summer to autumn. Our analysis kept plant as a random factor, and still there was a significant effect of insect group on rate.

Our plants were a mixture of self-compatible ones (bramble and marjoram [36–38]), self-incompatible ones [39], or a mixture of both/unknown [37, 40]. Additionally, during our study sometimes the insects visited multiple flowers on the same plants (e.g., California lilac), and sometimes the insects visited multiple, neighboring plants (ragwort, bramble, and marjoram). Therefore, we cannot calculate a pollination probability *per se* with these data [41, 42]; however, we have taken an important first step to quantify differences in behavioral patterns, specifically the speed at which different functional insect groups visit flowers. Lastly, pollinator efficiency of course would depend not only on visitation frequency, but also on the total number of visits from a given functional group (e.g., the density of an insect group). Our study, which was observational, demonstrates that insect density for our location varied greatly between functional groups, especially across the seasons (Figure 2), all of which would influence pollination.

Our census data shows that, overall, the honey bee was the most frequent insect found on all the plants (34% across all plants/locations; Figure 2), which is comparable to reported levels found in previous studies that determined honey bee proportions on spike lavender in Spain (24.8%) [2] or on garden plants in south England (28%) [43]. Although abundance will vary with location (i.e., proximity to apiaries of managed honey bee hives) and season, it is important to note that, in this study, only one location (Falmer) was near a known apiary (our laboratory). The study in Spain also reported that the nearest apiary was too far away to contribute significantly

to their reported honey bee abundance [2]. Rather, honey bees, whose colonies may possess tens of thousands of workers compared to the much smaller colonies of bumble bees or non-Apidae bees, may be just abundant in many Western landscapes. In addition to being abundant in numbers, honey bees are also abundant temporally throughout the foraging season: other insect groups appear in our census, peak, and then demonstrate a decrease in abundance, as dictated by their phenology. In contrast, honey bee colonies possess workers year round and for temperate climates usually have active foraging from early spring until late autumn [44]. Our study began at the end of June with the flowering of California lilac, which was followed by bramble, marjoram, ragwort, and finally ivy, which finished at the end of October. Honey bees were a consistent presence across the study (Figure 2). The honey bee and bumble bee are also in possession of another adaptation that most likely helps in their pollination efficiency. The hairiness of their bodies aides in the transfer in pollen [13, 15, 16]. This morphological adaptation, combined with their abundance and high flower visitation rate and, with honey bees, their long distance foraging [44], plays a role in making Apidae bees very effective pollinators.

Although we did not test why Apidae bees, like bumble bees and honey bees, are speedier as they visit flowers, it is interesting to consider a few options. One reason may be that honey bees and bumble bees only visit flowers to forage, whereas other insects, in addition to foraging, visit flowers to do other things like sun themselves or lay eggs [45, 46]. More work should be done to quantify and to compare per plant different time spent doing different behaviors per functional insect group. Future studies could investigate the contribution of life history differences on any foraging rate differences and may help our understanding of the complex way in which plants, insects, and environment interact within the process of pollination.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Ant *Cardiocondyla elegans* as Host of the Enigmatic Endoparasitic Fungus *Myrmicinosporidium durum*

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Data on host species and the distribution of the endoparasitic fungus *Myrmicinosporidium durum* increased continuously in recent decades. Here, we add the ant *Cardiocondyla elegans* as new host species. Colonies of the monogynous species were found infested in the region of Languedoc-Roussillon (South France). Samples from the nest indicate high infection rates. All castes and sexes were infected by the spores. Variations of infection rates between sampling methods and species are discussed.

1. Introduction

Ant-infesting parasitic fungi have been studied by scientists for more than a century. While the life cycles and transmission have been revealed for some fungal parasites, the endoparasitic fungus *Myrmicinosporidium durum* is only incompletely understood [1].

M. durum was first discovered in workers of *Solenopsis fugax* and *Temnothorax tuberum* and described as a haplosporidian protist [2, 3]. Only much later it was identified as a fungus [4]. Research over the last decades has broadened our knowledge about host species and global distribution but still little is known about its life cycle [3–5]. Up to now, almost 40 host ant species of the subfamilies Myrmicinae, Formicinae, and Dolichoderinae have been described [6, 7]. Infections have been reported from Western Europe to Asia and also from America (see [6]).

In France, *M. durum* has been reported first in 1982 by Espadaler and to date it has been found parasitizing four host species: *Temnothorax recedens* [8], *T. unifasciatus* [8], *Pheidole pallidula* [9], and *Solenopsis fugax* [9]. Generally, infested ants contain plenty of dark brown spores, which are lensoid in shape and measure up to 65 μm in diameter [4, 10]. Due to the characteristic shape of the spores, Hölldobler [3] described the infection as “Näpfchenkrankheit” (small bowl sickness). Spores initially occur in the gaster and later extend to other parts of the body [6, 7, 10] but never infest vital organs. Infected individuals therefore can still be long-lived [3, 4].

Most infected individuals were captured in pit fall traps or by hand in the field, indicating that infected workers forage. In addition, *M. durum* has been found in young swarming queens of *S. fugax* and a male of *P. pallidula* [11]; that is, both castes and both sexes of ants can be infested [10, 11] and at least workers and swarming queens continue to show their typical caste-specific behavior [3, 10]. The absence of detectable negative effects on the numerous hosts [3, 10] was interpreted as evidence for a long common evolutionary history of host and parasite [12].

Here, we report on the first finding of *M. durum* in workers, queens, and males of the ant *Cardiocondyla elegans* at the banks of Le Gard du Rhône, France. Colonies of the ant *C. elegans* are small with less than 500 workers, one queen, and in summer also numerous female sexuals and a few wingless (ergatoid) males [13, 14]. In our study area they nest in cavities in the soil. Sexuals mate in late summer in their natal nest and after hibernation young mated queens disperse on foot to found new colonies nearby [13, 14]. Our addition of a new genus to the already quite extensive list of host species supports the hypothesis that *M. durum* is a nonselective generalist with a large host range species [5–7].

2. Material and Methods

Study samples were collected at six different sites in the region of Languedoc-Roussillon (South France) from 19 to

TABLE 1: *Myrmicinosporidium durum* (endoparasitic fungus) infection rates in populations, colonies, and individuals of the ant *Cardiocondyla elegans*.

	Population	Number of colonies	Number of infected colonies	Colony infection rate (%)	Number of dead individuals	Number of dead infected individuals	Individual infection rate (%)
	C1	8	5	62,5	279	43	15,4
	C2	3	3	100,0	74	24	32,4
	C3	8	3	37,5	223	24	10,8
	C4	5	3	60,0	205	24	11,7
	R1	9	1	11,1	486	10	2,1
	R2	9	1	11,1	216	1	0,5
Total	6	42	15		1483	126	

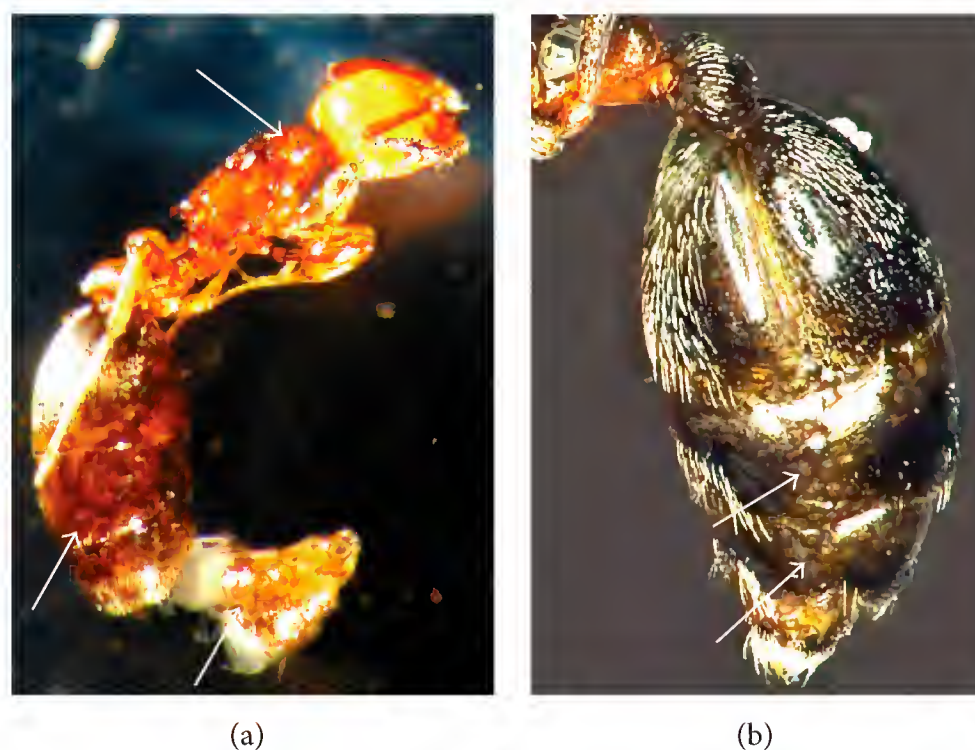


FIGURE 1: Male (a) and gaster of a worker (b) of *Cardiocondyla elegans* infected with spores of the endoparasitic fungus *Myrmicinosporidium durum*. Spores are easier visible in the lightly pigmented male compared to the dark worker.

28 September 2014 (Remoulins, R1: N43°56'31.1" E4°33'33.91"; R2: N43°55'45.296" E4°34'4.883"; Comps, C1: N43°50'38.314" E4°37'0.134"; C2: N43°50'56.364" E4°37'10.326"; C3: N43°51'9.889" E4°37'2.363"; C4: N43°49'34.561" E4°38'16.858"). Nests were located in moist and earthy habitats with grass patches and one site also contained sandy soil. Colonies were located by following foragers to the nest entrance. Nests were excavated, and ants were collected with an aspirator and transferred to the laboratory in Regensburg, where they were housed in plastic trays in incubators at 12 h light/12 h dark cycles. Temperature was gradually decreased from 18°C/13°C (day/night) to 15°C/10°C (day/night) and finally to 12°C/8°C (day/night) until mid-December and maintained for three weeks to simulate a short hibernation period. Afterwards, temperature was again gradually increased until mid-January (20°C/16°C day/night) and remaining queens and workers were separated for colony founding experiments. Colonies were fed regularly twice per week with honey and fruit flies or parts of cockroaches.

Colonies were scanned twice per week and all dead ants were stored in 100% ETOH and subsequently investigated

under a microscope. Infections were detected through the cuticle of dead ants with a microscope (magnification 35x). The dark spores of *M. durum* are easily detected in lightly pigmented species but hardly visible by eye in dark species [6, 7, 12, 15]. Female sexuals and workers have a dark coloration, while wingless (ergatoid) males, typical for *Cardiocondyla*, are yellowish [13, 14]. Hence, infections are much easier to recognize in males than in the female castes of *C. elegans* (see Figure 1).

3. Results and Discussion

Infected ants were found in all six investigated populations and in 15 of a total of 42 colonies (= 36.6%; for details see Table 1). Both, males and females (all castes) were found infested (see Figure 1). In most cases, spores were restricted to the gaster, but in several cases they also infested thorax and head. In our study, most of the infected individuals reared in the laboratory died before or during hibernation. After hibernation, only 4 of 137 (= 2.9%) dead ants were infected

with various developmental stages of the spores (observation period: mid-January until mid-March).

Our data suggest a rather high infection rate of *C. elegans* with the endoparasitic fungus *Myrmicinosporidium durum*. In previous studies, infection rates ranged between 0.17% and 66% [11, 15]. Furthermore, infection rates differ among species; for example, *Solenopsis fugax* had a much higher infection rate than *Temnothorax tuberum* [3]. This has been explained by the different colony size and behavior of the two species; in *S. fugax*, colonies are larger and, therefore, more workers are active and more likely to pick up spores and distribute them within the colony. In addition, workers of *S. fugax* are very active below ground, which may spread the fungal spores and cause a higher infection rate in the colony compared to the less active genus *Temnothorax* [3]. Generally, different infection rates may vary drastically, even within populations, dependent on the life cycle and the respective environmental conditions [6, 8], making it difficult to compare infection rates between and within species.

Interestingly, *C. batesii*, a close relative of *C. elegans*, was never infected although living in an area with other heavily infested ant species [6]. At the moment, we can only speculate about possible reasons. For example, colonies of *C. elegans* are larger and, hence, more individuals might forage and/or dig new nest chambers in the soil and thereby gather spores. Moreover, the special life history of *C. elegans* with an exchange of sexuals between colonies [13] might facilitate the spread of spores among colonies. Finally, different mechanisms of food filtration in the infrabuccal pocket of the ants might explain the interspecific variation in susceptibility [4, 16].

Unfortunately, it is still unclear whether *M. durum* is “[...] a generalist microparasite and not a mixture of differentiated host lineages” as suggested by Gonçalves and colleagues [6] or rather a fungus-complex [15] as it infests so many diverse ant subfamilies which are distributed over several continents [6, 7].

The increased mortality shortly after collection might be due to stress during excavation of the nest and transportation [15]. In contrast, under constant conditions, no reduction of lifespan has been reported [3, 4]. This finding is also supported by our study, as after hibernation, only a few infected individuals died.

4. Conclusion

Our study increases the geographic distribution and host range of *M. durum*. As the study species *C. elegans* can be easily maintained in the laboratory, it might be well suited for further investigations on the parasite's life cycle and transmission.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

An Insight into Sweet Potato Weevils Management: A Review

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Sweet potato is an important food crop that is grown widely in tropical and subtropical regions. Sweet potato weevil is the most disastrous pest affecting sweet potato plantations, causing millions of dollars losses annually. An effective integrated pest management (IPM) method will help to prevent economic losses, and it is crucial to understand the factors that contribute to weevil infestation and strategies that are available to overcome them. This review summarizes the (1) mechanisms of action of weevil on sweet potato and (2) contributing factors in weevil infestation, followed by (3) discussion on current IPM practices used in the different regions, including intercropping, entomopathogenic fungi and bacteria, sex pheromones, and pesticides. Lastly, it also focuses on (4) applications of advanced biotechnology and genomics strategies towards reducing weevil's infestation in sweet potato plantation.

1. Introduction

Sweet potato is currently ranked as the seventh most important crop in the world with a total production of 103 million tonnes in 2013 [1]. It is produced largely in Asia (accounting for up to 76.1% of world production in 2013), followed by the African continent (19.5%) [1]. The top five producers of sweet potato in 2014 were China, Nigeria, Uganda, Indonesia, and the United Republic of Tanzania [1]. Sweet potato is one of the five most important crops in 40 developing countries besides rice, wheat, maize, and cassava [2]. Despite the crop's economic importance, widespread sweet potato weevil infestation results in losses of millions of dollars annually [3].

Since weevils are widely dispersed in tropical regions of the world, their management is the key issue faced by farmers in major sweet potato producing countries. The four main species of weevils that cause the most harm to sweet potato plantation are *Euscepes postfasciatus* (Fairmaire), *Cylas formicarius* (Fabricius), *Cylas puncticollis* (Boheman), and *Cylas brunneus* (Fabricius) [4, 5]. *Euscepes postfasciatus* is a South American species that is more prevalent in Central and South America. *Cylas formicarius* is an Asian species but is usually found throughout the tropical regions worldwide including North America, the Caribbean, Europe, Africa,

Asia, and Oceania. *Cylas brunneus* and *Cylas puncticollis* are African species and are restricted to Africa. There are other species of sweet potato weevils in the tropical regions in Africa, for example, the rough sweet potato weevil (*Blosyrus* spp.) and striped sweet potato weevil (*Alcidodes dentipes* and *Alcidodes erroneus*), but their damage to sweet potato cultivation is not as severe as the main species (*Cylas* spp.) [6].

1.1. Sweet Potato Weevil Infestation by Regions. Sweet potato weevil is by far the most destructive pest of the sweet potato plant. The degree of infestation varies from region to region but weevils nevertheless cause severe damage to plantations. A research conducted by the Taiwan Agricultural Chemical and Toxic Substances Research Institute Council of Agriculture revealed that the damage caused by sweet potato weevil reduced sweet potato production in Taiwan by 1–5%, while the damage in extensive commercial produced fields was up to 18% [7].

In China's Guangdong Province, yield generally shrunk by 5–20% and, in some cases, by up to 80% [8]. In Vietnam, farm-level plantations losses were documented to suffer up to 40% of reduction in yield [9]. Losses of 3–80% were recorded in Indonesia, throughout several locations and seasons [10] and with higher damage observed during the dry season [11].

In Malaysia, exact losses in the field were not recorded in recent years; the only documented figure was a 1970 study showing 80% yield loss or 4 tons/acre [12]. In the Philippines, sweet potato yield was reduced by 50% due to *C. formicarius* infestation [13], while in Japan (Amami Islands), losses were recorded at 15% [14].

Cylas spp. were found to be the major pest in the tropical regions in Africa. Losses were recorded to be as much as 73% in Uganda [15] depending on the planting period and 15–20% in yield in Tanzania [16]. In other areas in the continent, losses have been shown to reach up to 100% [17, 18].

In the United States, the southern states of Alabama, Louisiana, Mississippi, and North Carolina produce 75% of sweet potato supply [19]. While the main pests affecting sweet potato plantations in these regions include sweet potato weevils, wireworms, white grubs, sweet potato flea beetle, cucumber beetle, white fringed beetle, and sugarcane beetle, weevils cause the worst damage to this crop [19]. In Southern Florida, up to 80% (average 69%) of losses in sweet potato yields have been reported, mainly contributed by the death of infested plants [20]. Due to the severity of this problem, several workshops were conducted by universities in this state to bring together all parties involved to develop plans for pest management in sweet potato production [19]. Through these activities, IPM strategies were implemented (such as pheromones trap and usage of selected approved pesticides); farmers were also given information about planting strategies (bedding and soil preparation) to minimise infestation and the introduction of sweet potato cultivar with higher resistance to weevils (such as Regal) [21].

In the Dominican Republic, sweet potato farm-level losses due to weevil infestation were estimated at 39% [22]. Weevil is also found in all provinces of Cuba where sweet potato plantations are located and, in the absence of adequate control, losses have been shown to reach up to 45% [23].

1.2. Sweet Potato Weevil Mode of Action. Although different subspecies of sweet potato weevils can be found in different geographical locations, their modes of action remain the same [24]. Sweet potato weevils generally cause serious damage to all parts of sweet potato plant throughout their life cycle, from egg to adult. When laying eggs, female weevils excavate cavities and create egg-laying punctures in the roots. The eggs are laid below the surface of the roots and covered with dark colour excrement from the female adults [24]. As a result of the unsightly punctures, the appeal of the roots and market price of sweet potato become greatly reduced, resulting in major economic losses.

Hatching will generally occur in a week after oviposition by females. Hatched larvae will start making tunnels inside tubers and feed inside galleries [25]. The tunnels inside the tubers of sweet potatoes will be filled with excrement from the larvae. As the larvae feed, the sweet potato will impart a bitter flavour and terpene odour, making it unsuitable for the consumption of human or livestock. The presence of terpenoid reduces the marketable yield and root quality of sweet potatoes [26]. Mining of sweet potato tubers by larvae is the principal cause of sweet potato damage. The tuber becomes spongy in appearance, riddled with cavities, and dark in

colour [26]. Beside that, larvae also mine into the vines of sweet potato, causing it to darken, crack, and collapse. Apparent symptoms of weevils infestation will be yellowing of the vines but this usually only occur after heavy infestation [26].

Larvae tunnelling inside the tubers will indirectly facilitate the entry of soilborne pathogens, which can cause further damage from secondary infections by fungi and bacteria [25]. In addition, the larvae will cause damage to the vascular system, which then reduces the number and size of future storage roots. Subsequently, adults will start emerging and will start feeding on leaves, vines, tender buds, and storage roots of sweet potato by punctuating the surface. Since most of the infestations generally occur below the soil level, these problems can go undetected until harvesting season arrives. Observations from the different regions of sweet potato plantations summarize the factors that can contribute to sweet potato weevil infestation. This information is important as a preventive measure against future infestation.

2. Factors Influencing Sweet Potato Weevil Infestation

2.1. Physical Attributes of Sweet Potato. Host plant resistance plays an important role in the management of serious insect pests [27]. Apart from the nutritional quality of its tuber, the physical attributes of sweet potato, including its flesh colour, neck length, shape, thickness, and skin colour, influence the infestation by sweet potato weevils. Oval- and round-shape sweet potato tubers were more severely infested by sweet potato weevils compared to elongate, spindle, and long stalked ones. Besides, cultivars with pink and red coloured tubers as well as lobed leaves and thin foliage were considered less susceptible compared to brown and white coloured tubers [28].

The level of infestation in the different sweet potato genotypes was reported to be related to the concentration of kairomones in the periderm of the tubers. For example, boehmeryl acetate, a kairomone identified in sweet potato tubers surface, acts as an ovipositional stimulant for female weevils [29]. This finding was supported by Nottingham et al. [30] who discovered triterpenol acetate on the root surface of the genotype “Centennial” which has shown similar function to other kairomones. This suggests that selection of sweet potato genotypes with increased deterrents or decreased concentration of kairomones such as boehmeryl acetate and triterpenol acetate may significantly facilitate sweet potato resistance to weevils [27]. Therefore, the selection of sweet potato variety is important for the control of sweet potato weevils.

Deep-rooting and early maturing varieties (90 to 120 days) are about four times less susceptible to infestation than shallow-rooting and late maturing varieties (180 days or more). As a result, both deep storage roots and early maturing varieties tend to reduce the severity of weevil damage [31]. More than 95% of oviposition by female weevils happens in the first 35 cm of vines and planting of infested cuttings is one of the ways of distributing sweet potato weevils [31]. Therefore, treatment of infested stem cuttings

with insecticides or *Beauveria bassiana* is currently being practised to reduce weevil infestation.

2.2. Age of Stem Cutting. Female weevils tend to lay eggs in the older portions of vines, especially when they cannot access the roots or the storage roots are absent. Since planting of infested cuttings or vines will spread weevil infestation, weevil-free cuttings of sweet potato vines produced by dipping in an insecticide solution are recommended [32]. Older portions of cuttings are usually severely infested with weevils, while younger cuttings are rarely infested with weevils. This notion is supported by a field study, which showed increase in number of weevils in vines in increased vine age [33].

2.3. Altitude and Season. Weevil infestation has a strong relationship with the location altitude and planting season of sweet potato. Several studies have concluded that higher temperature may increase the growth rate of insect's population as well as the risk and severity of the outbreaks [34, 35]. In Kerala, India, tuber damage by weevil infestation was observed to be less serious in lowland (up to 22%) compared to upland (4 to 50%) [36]. On the other hand, a study in Kabale District, Uganda, reported higher number of *Cylas* spp. infestation at lowland (up to 1814 meters above sea level) (77%) compared to the number of *Cylas* spp. at higher altitude (1992–2438 meters above sea level) (23%) [37]. The rate of infestation in this case may be also affected by other reasons such as method used in planting, sanitation level in that area, and the variety of sweet potato planted.

According to Bhat [38], the incidence of weevil infestation was higher when planting in the period from August to November (87.4%) compared to the planting in the period from June to July (10.9%). A study conducted in India showed that tuber damage was higher (71%) during summer season (February–May) compared to monsoon season (June–September) (45%) [36]. Thus, weevil infestation can be reduced by proper planning of planting and harvesting time as well as the planting location.

In addition, sweet potato weevil relies strongly on cracks in dry soil to reach the storage roots, as they cannot dig. Hence, weevils cannot reach roots which are well buried under the soil. The enlargement of roots near the soil surface and the stress from soil moisture can increase the chances of producing cracks and exposure of roots to the weevils. The incidence of damage caused by sweet potato weevils was observed to be lesser during wet season compared to dry season as the absence of cracks hinders weevils from accessing the roots [39–41].

3. Current Strategies to Overcome Sweet Potato Weevil Infestation

3.1. Mixed Cropping or Intercropping. Some farmers practise mixed cropping systems with sweet potato, rice, cowpea, maize, ginger, and yam to reduce the incidence of sweet potato weevils. Pillai et al. [42] reported that intercropping sweet potato with colocasia, rice, or cowpea resulted in up to tenfold reduction in the infestation of sweet potato weevil

(4.8–11.54 weevils per kg of tubers) compared to monocrop of sweet potato (217.5 weevils per kg of tubers) in Kerala, India.

Besides, effective crop rotations also resulted in lower tuber damage (ranged from 7 to 9%) compared to monoculture of sweet potato (52%) [43]. Crop rotation method was found to be effective in controlling weevil infestation as the number of weevils captured by sex pheromone traps in areas with monoculture of sweet potato exceeded exponentially compared to pheromone traps in areas planted with sweet potato rotated with potato [44].

Additional steps such as elimination of crop residues will also increase the effectiveness of IPM. For instance, a field sampling in Cuba showed that 0.7 tonnes of crop residues per hectare could harbour approximately one million weevils. Aside from this, new sweet potato plantings are also recommended to be separated from older fields (approximately 1 km) as the risk of weevil infestation increases with the age of sweet potato fields. This distance length was chosen as the estimated dispersal ability of the weevils from invading a new plantation [45, 46]. In Japan, alternative crops were suggested to farmers to be planted between sweet potato plantations, for example, yam (*Dioscorea japonica*), “senryo” (*Chloranthus glaber*), and edible sunflower [47].

3.2. Mulching, Irrigation, and Reridging. Since dry soil allows weevil to reach the target roots, an important strategy to deter infestation is by preventing soil cracking. This can be achieved by irrigating frequently or hilling a small area around the sweet potato plant in order to prevent the entry of weevils into the roots. An experiment was conducted by Talekar [48] to determine the potential of mulching materials such as rice straw and plastic film in reducing the infestation of sweet potato weevils by spreading them over the planting site of sweet potato. The result showed that rice straw and plastic film successfully reduced weevil infestation by 55% and 35%, respectively. Mulching materials were found to minimize soil cracking and conserve soil moisture and provide a physical barrier that reduced the entry of weevils to roots.

Reridging is another approach that works to prevent the entry of weevils into tuber and oviposition by female weevils, but this works best only when performed at the tuber formation stage. Palaniswami and Mohandas [49] conducted a study in India to investigate the efficiency of reridging in sweet potato plant in reducing the infestation of weevils. It was observed that the weevil infestation was significantly reduced by this method.

Soil cracking due to deficiency in irrigation or drought will facilitate the entry of eggs into the roots. Therefore, in Cuba, sweet potato was commonly planted during rainy season because less irrigated fields were 4 to 5 times more infested with weevils compared to well-irrigated fields. Besides, weevil associated damage also increase by over 4 times if harvesting was delayed by 30 days; hence, it is necessary to harvest mature crops before the infestation level reached 3% [50, 51].

3.3. Sanitation. Sanitation practices play a vital role in protecting sweet potato from pests with limited flying capacity such as sweet potato weevil. Weevils that survived in stems

and roots will infest neighbouring sweet potato plants. To overcome this problem, crop residues in the field must be destroyed after harvest [52]. Traditionally, infested sweet potato fields were flooded to induce the rotting of leftover plant materials and to reduce the chances of weevil infestation in neighbouring plants. However, it was only in 1987 that experiment to study the effect of flooding of infested fields on the weevil densities in sweet potato plantation was conducted [47]. This was conducted by maintaining standing water over recently harvested weevil-infested sweet potato fields for approximately 4 weeks. The results showed that the number of insects (including larvae, pupae, and adults) per plant decreased over the flooding duration from 1 week to 4 weeks, demonstrating that flooding could be a safe and cost-effective method to reduce weevils infestation in sweet potato plantation.

3.4. Entomopathogenic Nematodes. Biological control method using entomopathogenic nematodes has been found to have beneficial interaction with sweet potato and offers a promising way to suppress sweet potato weevil population. The entomopathogenic nematodes, *Steinernematidae* and *Heterorhabditidae*, have high reproductive potential and are safe to nontarget organisms, two characteristics which make them attractive for use against pests such as sweet potato weevils [53].

Infective juvenile entomopathogenic nematodes penetrate the body cavity of larvae via the mouth or breathing pores by using a tooth-like structure that pierce into the host soft intersegmental membranes [54]. Once inside, the nematodes start releasing pathogenic bacteria, *Photorhabdus* by *Heterorhabditidae* and *Xenorhabdus* by *Steinernematidae*, into the larvae body cavity, where the bacteria will reproduce rapidly and cause larvae death within two days [55, 56]. Mutualistic relationship exists between the bacteria and nematodes in which the bacteria supply nutrients to the nematodes by killing the insect host, while the nematodes provide shelter to the bacteria [57].

The use of entomopathogenic nematodes in controlling sweet potato weevils offers several advantages: they are environmentally safe, infective juveniles can be used together with most pesticides [58, 59], and they are host-specific [60]. Jansson et al. [61] conducted an experiment to test the effect of entomopathogenic nematodes on *C. formicarius* and the results showed that the application of entomopathogenic nematodes (*Heterorhabditidae bacteriophora*) consistently reduces damages to sweet potato roots and is thus suitable to control weevil infestation.

3.5. Entomopathogenic Fungi. Entomopathogenic fungi are by far one of the most effective biological control agents of sweet potato weevil due to their host-specificity. Entomopathogenic fungi are one of the first organisms involved in biological control of pests and recently more than 700 species are found to be pathogenic to insects [62]. These fungi infect their hosts by entry into the host, followed by evasion of host defence reactions and multiplication and finally exit from the host [63]. The physicochemical features of disease development require interaction of entomopathogenic fungi

with the insect host's outer tissues before producing toxic metabolites to evade or interact with the insect's defense mechanisms, though some metabolites were found lacking this ability [64, 65].

Several mechanisms are used by entomopathogenic fungi to evade the insect host defense system within the hemolymph, including changes in outer cellular layer of the fungi and the production of immunomodulating substances to suppress host defense system [66, 67]. Entomopathogenic fungi produce a relatively high level of toxic metabolites, enabling them to tolerate the insect's immunological defense system. For instance, *Beauveria bassiana* produces 10 kDa of a proteinaceous metabolite to kill the larvae at metamorphosis stage by disrupting the granulocytes [68]. The death of the insect host usually is a result from depletion of nutrient resources, mechanical damage, and toxicosis [62].

Protein contributes 70% of the cuticle layer; thus proteases secreted by entomopathogenic fungi play an important role in digesting protein and penetrating the cuticle layer [69, 70]. The cuticle degradation by proteases starts with the absorption of enzyme onto the cuticle by nonspecific electrostatic bonds, followed by protease active site contact with specific peptide sequences (on the cuticle) and lastly the hydrolysis of amino acids or peptide fragments of the cuticular protein. Besides, entomopathogenic fungi also secrete lipoxygenases, phospholipases, and lipases in order to degrade the host epicuticle which contains fats, lipoprotein, and waxy layers [71]. These three enzymes hydrolyze ester linkages in glycerophospholipids of host cuticle and result in cell lysis, destabilization of membranes, and release of second messengers [72].

Numerous studies and laboratory experiments have proven that entomopathogenic fungi are useful in the control of sweet potato weevil. Reddy et al. [73] conducted a field study to compare the effectiveness of entomopathogenic fungi, insecticides, and combination of both entomopathogenic fungi and insecticide in controlling sweet potato weevil by determining the adult weevils' mortality. Results showed that *Metarhizium brunneum* with spinosad (insecticide) and *Beauveria bassiana* with spinosad caused 100% adult weevil mortality at 48 hours after treatment, while *Metarhizium brunneum* and *Beauveria bassiana* alone required 168 to 192 hours after treatment to cause 100% mortality. Beside that, Ondiaka et al. [74] conducted an experiment to determine the effect of *Beauveria bassiana* and *Metarhizium anisopliae* on adult *Cylas puncticollis* and fecundity and viability of female *Cylas puncticollis* eggs. The results showed that spraying of *Beauveria bassiana* or *Metarhizium anisopliae* caused adult mortality between 62.5% and 89.2%. In addition, adult females treated with both fungal species laid less eggs compared to control, suggesting that the fungi can reduce fecundity and egg viability significantly.

3.6. Bacterial Insect Pathogens. Bacteria insect pathogen can grow, multiply, and develop within an insect host, causing considerable damage to the host. Bacteria enter insect host through the oral cavity or outer integument, which consists of cuticle and epidermis. Bacteria need to overcome several threats after they have entered the insect host via the oral

cavity, and these include high concentration of hydrogen ion and antibacterial substances in plant (insect food) and low oxidation-reduction potential in gut, which restrict the proliferation of bacteria. The bacteria establish themselves by penetrating epithelial cells of the gut and invading host hemocoel, which will eventually reduce the nutrients in the host gut, causing starvation or sickness to the host [75].

Biocontrol agents are generally divided into three main groups: Group 1—agents will recycle naturally to exert permanent effect of pest control once they are introduced into the pest population; Group 2—agents disappear quickly from the pest population and they must be reapplied regularly; and Group 3—agents can behave like Group 1 or Group 2 based on the combination of pest species, environment, and agent strains. Among the most commonly used bacteria in pest management, industrially produced *Bacillus popilliae* falls into Group 1, which has long-term economic control on pests. On the other hand, industrially produced *Bacillus thuringiensis* falls into Group 2, which has practical but transient control of pests, and lastly *Bacillus sphaericus* falls into Group 3, which has mixed characteristics of Groups 1 and 2 [76].

Group 1 bacteria produce spores near the soil surface. Larvae, which feed on the roots below soil surface, will eventually consume the bacterial spores that have contaminated their food. The rate of disease development depends on temperature, spore size, and spore dosage. Long-term parasite-host balance can be ensured due to the absence of toxins, involvement of vernalization, and the complexity of Group 1 bacterial spores [77]. Group 2 bacteria produce crystal toxin, which attacks host gut wall, impairing enzyme secretion and lowering the pH of the gut so that the spores could germinate [78]. The multiplication of Group 2 bacteria in the host gut results in mouth paralysis, septicaemia, starvation, larvae death, reduced egg production, infertility, and bacteria sporulation. However, the application of Group 2 bacteria as outdoor microbial insecticide possesses several disadvantages such as the inability to spread widely among pest populations, rapid disappearance, and being easily destroyed by solar radiation [79]. On the other hand, partial digestion of Group 3 bacteria will release toxin after ingestion of spores and the toxin will penetrate into the peritrophic membrane before expressing its toxicity. Group 3 bacteria will usually multiply rapidly, sporulate, and infect healthy larvae after the disintegration of cadaver [80].

The application of bacteria in controlling pests offers several advantages including long shelf life of bacterial spores, high resistance to unfavourable environment, and small size that permits effortless design as chemical pesticides. Furthermore, bacteria are safe to nontarget organisms including beneficial flora and fauna, have low production cost, and do not require safety precautions during their application [75].

3.7. Sterile Insect Technique. Irradiation of sweet potato weevil with gamma rays is known as sterile insect technique (SIT). It is frequently used for the sterilization of weevil in tubers meant for long storage or export purposes [81, 82] and for the release of irradiated weevils into environment for eradication programmes [83].

The sterile males will vastly outnumber wild males and compete for the females in mating. The mating of sterile males with females will not produce offspring, resulting in a dramatic reduction in the next generation population [84]. Other than affecting fertility, the increase in absorption of radiation also causes negative effects on somatic cells and reduces the insect quality [85]. For instance, radiation damages the midgut epithelium, which disrupts the insect's nourishment process [86].

Setokuchi et al. [87] conducted a long-term experiment to investigate the efficiency of sterile insect release method to eradicate *Cylas formicarius* in the field. They irradiated weevils with 80 Gy, stained them with fluorescent dyes, and released them in a release zone in 1994 and 1995. The weevils were monitored using pheromone traps and root traps (1994 to 1995) and the effect of sterile weevil release was reexamined in 1996. The results showed that the number of unmarked weevils caught in the release zone was reduced to nearly zero in 1995 and no living weevils were captured in both pheromone and root traps during the reexamination in the release zone in 1996. This showed that sterile insect release method is useful in controlling sweet potato weevil infestation.

More recently, Kumano and colleagues [83] have conducted experiments to determine the irradiation effect (200 Gy dose) on the mating ability in *Cylas formicarius elegantulus* male by comparing the longevity, mating performance, and mating competitiveness with the control male. The results showed that the survival of male weevils decreased drastically after being irradiated with gamma rays. Beside *C. formicarius*, SIT has also been used to target *E. postfasciatus* (West Indian sweet potato) but due to the lack of effective attractant, the dispersal activity of this pest is currently limited [46].

To sum up, sterile insect technique is a viable method to reduce weevil population in sweet potato plantation. However, the effect of their release into the environment in the long term, ecosystem consequences, effects on ecological needs, and potential of resistance should be well studied before this method can be widely practised [88].

3.8. Chemical Control. Various synthetic chemical insecticides are currently used in sweet potato plantation to prevent or treat sweet potato weevil infestation. Organophosphates chlorpyrifos and imidacloprid, which are chloronicotinyl insecticides, act primarily on the insect central nervous system by binding irreversibly to insect nicotinic receptor, leading to nicotinic neuronal pathway obstruction and eventually failure in production of acetylcholinesterases. Acetylcholinesterases are required to break down or deactivate acetylcholine in chemical synapse. The lack of this enzyme will result in accumulation of acetylcholine, overstimulation of cholinergic synapses, paralysis, and eventually the death of the insect [89].

Mason and Jansson [90] conducted an experiment to compare the toxicity of five insecticides: parathion, carbamate methomyl, chlorpyrifos, chlorinated hydrocarbon endosulfan, and carbamate carbaryl, against adult *Cylas formicarius* using Petri dish bioassays in laboratory. The results showed

that organophosphates parathion and chlorpyrifos were the most toxic as they had the lowest LD₅₀ values (1.97 and 5.12 µg/g of wet biomass), followed by methomyl (6.03 µg/g of wet biomass), endosulfan (57.44 µg/g of wet biomass), and lastly carbaryl (297.41 µg/g of wet biomass). Due to their higher toxicity, chlorpyrifos and parathion were suggested for the control of sweet potato weevils. In a separate study, Hwang and Hung [91] conducted a field experiment to test the efficacy of five insecticides: chlorpyrifos, phorate, terbufos, fensulfothion, and carbofuran, in controlling sweet potato weevils, by applying the insecticide twice to soil before planting and during earthing up. The results showed that chlorpyrifos had the highest rate of control (76.8%), followed by fensulfothion (51.3%), phorate (44.9%), carbofuran (38.8%), and lastly terbufos (38.0%). In both studies, chlorpyrifos demonstrated a high efficacy in suppressing sweet potato weevil infestation and hence it is widely used in the integrated pest management of this pest.

3.9. Sex Pheromone Trap. Sex pheromone trap is widely used to capture adult sweet potato weevil in plantation owing to its high efficiency [92]. The trap is usually designed with synthetic pheromone lure such as (Z)-3-dodecen-1-ol (E)-2-butenate together with ethyl acetate and is usually placed at ground level to facilitate the entrance of adult weevils, which will then be killed by the insecticide inside the trap. Pheromones are used in three ways: mass trapping to reduce insect pest population, insect populations monitoring using pheromone traps, and mating disruption by applying high dosage of pheromone in the atmosphere [92].

(Z)-3-Dodecen-1-ol (E)-2-butenate, isolated from female sweet potato weevils and synthesized chemically [93], is a successful mating disruptant of sweet potato weevils [94]. This novel compound provides several economic applications such as the detection of weevil outbreaks, the monitoring of an existing weevil population to schedule eradication programmes, and the control of mating in adult populations by attracting a large portion of male weevils or by disruption of mating through inhibitory properties [94].

Reddy et al. [95] conducted a field study in Guam to determine the efficacy of controlling *Cylas formicarius* using bucket traps with (Z)-3-dodecen-1-ol (E)-2-butenate. Their effectiveness was measured by the total damages caused by weevil and the total sweet potato yield for that season. The results showed that sweet potato roots damage in both locations with traps was very low (< one feeding hole per root) from June to September, compared to locations without traps (approximately 38 feeding holes per root) during the same duration. The sweet potato yield in fields with traps was higher (13.47 tonnes per hectare at Inarajan and 14.59 tonnes per hectare at Yigo) compared to fields without traps (7.86 tonnes per hectare at Ija and 8.26 tonnes per hectare at Dededo), proving that pheromone traps are effective in reducing the damage done by sweet potato weevil due. Reddy et al. [96] also conducted a study to evaluate four parameters (size, trap colour, trap design, and height of traps placement) that influence the effectiveness of sex pheromone trap used together with (Z)-3-dodecen-1-ol (E)-2-butenate. The result showed that medium-sized red Pherocon (USA) unitraps

(13 cm × 17.5 cm) were more effective to increase the efficacy of sex pheromone traps for sweet potato weevils.

In a separate study, Smit and colleagues [97] conducted field experiments to determine the efficacy of mass trapping of *C. brunneus* and *C. puncticollis* by using decyl (E)-2-butenate and dodecyl (E)-2-butenate, one of the important components of adult sweet potato female weevil sex pheromones. The traps, baited with 0.1 mg of decyl (E)-2-butenate and dodecyl (E)-2-butenate, were placed in 0.5-hectare fields. Four trials with durations ranging from 31 to 37 weeks were carried out. The results showed that a maximum reduction in the population of male *Cylas brunneus* and *Cylas puncticollis* (89%) was achieved from the four trials. This proved that pheromone traps baited with female sex pheromone could reduce the male sweet potato weevil population effectively and ultimately reduce the chances of mating in the population.

4. The Future of Sweet Potato Crop

Advancements in molecular biology tools including Next Generation Sequencing have provided multiple advantages in the understanding and study of economically important crops. Currently, the International Potato Center (CIP), the main research organization working on root crops, is collaborating with the University of Ghent to determine the sequence the whole genome of *Ipomoea batatas* cv. *Huachano* using SOLiD complete genome shotgun sequencing. The lack of whole genome sequencing data of sweet potato has hindered DNA fingerprinting, development of molecular markers, and genetic analyses of this crop. DNA fingerprinting is important in many crop species for the identification of crops to protect the rights of plant breeders. Monden et al. [98] had conducted DNA fingerprinting based on the two active retrotransposon families (Line-type L1b and LTR-type Rtsp-1) in sweet potato, which exhibited high insertion polymorphisms, by using the MiSeq sequencing platform. The polymorphism of these insertion sites was very high (91.4%) and the insertion sites, which are cultivar-specific, were successfully converted into amplified region markers. The markers helped in the precise identification of sweet potato cultivars.

Tao et al. [99] conducted a transcriptomic study on sweet potato to further understand the molecular mechanism and gene expression in different tissues at different developmental stages as well as the biotic and abiotic stress responses in sweet potato. From this study, a large number of genes were found responding to drought, salt, heat, and osmotic stress such as those encoding metallothionein (MT), aquaporin (AQP), and abscisic acid responsive elements binding factor (AREB). Understanding the expression patterns of these genes could possibly lead to a stronger crop in the near future. Prior to this, two other sweet potato transcriptomes were sequenced by the International Potato Center and the Guangdong Academy of Agricultural Sciences of China using the Roche-454 pyrosequencing technology [100] and the Illumina/Solexa RNA-Seq technology [101], respectively. These studies have led to new paths in collating differentially expressed genes in the different tissues at the different development stages. This will also allow better understanding

of the different genes involved in the pathways and provides information for further modification in gene expression to improve the quality of the crops.

Aside from this, development of orange-fleshed sweet potato with high level of beta-carotene is seen as a strategy to improve the nutritional status of millions of children in developing or underdeveloped countries. However, conventional breeding of orange-fleshed sweet potatoes are difficult due to their genetic complexity, thus marker-assisted breeding tools must be developed to assist in their breeding. A study was conducted to determine the quantitative trait loci (QTL) for starch, beta-carotene, and dry matter content in sweet potato [102]. The identification of QTL was based on the hexaploid sweet potato mapping population that resulted from a cross between Beauregard and Tanzania sweet potato cultivars in USA. QTL analysis in two parental maps [103] revealed 12 QTL for starch content, 8 QTL for beta-carotene, and 13 QTL for dry matter content in storage root. The results improve our understanding of these important traits in sweet potato, which are important for the development of marker-assisted breeding tools to increase sweet potato breeding efficiency.

The development of transgenic sweet potato with foreign genes that confer additional properties such as insect resistance is important to reduce economic losses due to pest infestation. The progress of breeding sweet potato with weevil resistance is slow due to scarcity of sweet potato varieties that display significant resistance levels [104], hindering progress in this area. Most of the early works focused on the transformation of sweet potato with proteins that decrease sweet potato digestibility for insects. Newell et al. [105] transformed sweet potato with mannose binding snowdrop lectin and cowpea trypsin inhibitor. Both transgenic sweet potatoes showed a moderate increase in resistance of sweet potato weevil. In a separate work, Cipriani et al. [106, 107] transformed soybean Kunitz type trypsin inhibitor and rice cysteine proteinase inhibitor into sweet potato. However, the transformation works, which focused on proteinase inhibitors, were abandoned due to concerns regarding nutritional impact of the proteins on human diet and after observing a small increase in weevil resistance against the proteinase inhibitors [108].

A number of studies have supported the high efficiency usage of *Bacillus thuringiensis* in controlling different pests including sweet potato weevils. García et al. [109] transformed the stems and leaves of sweet potato with *Agrobacterium tumefaciens* carrying nptII and *Bacillus thuringiensis* (Bt) endotoxin gene. The plants were then tested for resistance against sweet potato weevil under controlled conditions. Their results showed that the transgenic sweet potato carrying Bt gene had higher resistance compared to control plants (weevil damage on the normal sweet potato was five times higher compared to transgenic). The transformation of crops with *Bacillus thuringiensis* Cry proteins showed promising results in pest control through the expression of insecticidal compounds. Morán et al. [110] conducted a study to transform Cry3A gene into sweet potato roots to fend against sweet potato weevil, *Cylas formicarius*. The transformed sweet potatoes were able to express Cry3A protein but this research was discontinued, as the control of *Cylas formicarius* with

Cry3A was not promising. Ekobu et al. [111] conducted an experiment to evaluate the toxicity of Cry proteins to *Cylas puncticollis* and *Cylas brunneus*. They showed that three Cry proteins: ET33/34, Cry7Aa1, and Cry3Ca1 had LC₅₀ below 1 µg/gram diet, thereby proved that these transformed sweet potatoes can be used against weevil infestation.

Other biotechnological approaches have also shown promising results on pest control, including introduction of small RNA viruses that interfere with weevil life cycle [112], spider venom toxins [113], and RNA-mediated interference [114]. RNA-mediated interference promotes posttranscriptional gene silencing and has been used in insect management and virus disease resistance development in sweet potato. For example, Kreuze et al. [115] conducted an experiment to develop sweet potato with RNA silencing-mediated resistance to sweet potato chlorotic stunt virus. They found that 50% of the tested transgenic events showed mild or no symptoms of infection of the virus and the accumulation of the virus in the transgenic sweet potato was significantly reduced.

In all, the understanding of the biology of sweet potato weevil infestation is essential so that specific preventive method can be designed. Numerous strategies and techniques have been used in the different regions; however, until recently, they only serve to suppress the potential damages by the weevils without addressing the underlying problem. The momentum in biotechnology research field has given the sweet potato industry a promising future. Despite the absence of transgenic weevil resistance sweet potato in the market, hopefully, the various research teams currently working on this can release the long-awaited transgenic sweet potato to consumers soon.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Taxonomy and DNA Sequencing of *Crematogaster coriaria* Mayr, 1872 (Hymenoptera: Formicidae), with Redescriptions of the Worker, Queen, and Male Castes

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Crematogaster coriaria Mayr, 1872, is revised based on the nest series samples. The following new synonymies are proposed: *C. coriaria* Mayr, 1872 = *C. coriaria fraudatrix* Forel, 1911 *syn. nov.* = *C. spengeli* Forel, 1912 *syn. nov.* = *C. spengeli taipingensis* Forel, 1913 *syn. nov.* Worker, queen, and male castes of *C. coriaria* Mayr, 1872, are redescribed. The polymorphism of *C. coriaria* Mayr, 1872, was also discussed. DNA sequences were generated for *C. coriaria* Mayr, 1872, using an amplification of second half region of the mitochondrial gene COI with a total of 711 bp. The interspecific COI divergence is 17.4% for *C. coriaria* Mayr, 1872, and *C. modiglianii* Emery, 1900.

1. Introduction

Crematogaster coriaria Mayr, 1872, was described by Mayr [1] based on the worker specimens from Borneo, Malaysia. One following subspecies of *C. coriaria* Mayr, 1872, has been recognized: *C. coriaria fraudatrix* Forel, 1911, by Forel [2] based on the workers from Sumatra, Indonesia. This species had been classified into the former subgenus *Paracrema* but presently assigned to the subgenus *Crematogaster* [3].

Crematogaster coriaria Mayr, 1872, is distributed in Malaysia (Peninsula, Borneo) and Indonesia (Sumatra, Java) and has been reported from several localities in the literatures [4–6]. This species inhabits well-developed forests and nests in the dead woods.

This species is relatively roughly distinguished from other *Crematogaster* fauna by 4-segmented antennal club and weak sculptured body surface. However, due to the strong polymorphism, the taxonomy of this species has been confused as follows. This species is distinctly polymorphic in size and sculpture, causing some synonymies in some species and subspecies. *Crematogaster coriaria* Mayr, 1872, has relatively smooth surface of body in smaller workers but develops the rugulae and sculpture in larger workers.

The present paper aims to revise the synonymies based on the nest series samples. Such samples can reveal morphological continuities within conspecific taxa.

As in other ant genera, taxonomy of the genus *Crematogaster* has been generally improved based on the worker caste [7]. The sexual forms are poorly investigated because of the rarity of sexual castes in the collections and unexplored characters in the genus (but see [8, 9]). The present paper describes queen and male castes of *C. coriaria* Mayr, 1872, and it will provide a starting point for taxonomic studies to find the valid characters.

Molecular approaches including DNA barcodes have been recently used in biodiversity studies of ant species [10] and are used as an additional and powerful method in integrative taxonomy [11]. They can thus provide a useful reference for the identification of Asian *Crematogaster* species. Therefore, cytochrome oxidase I (COI) sequence data from *Crematogaster coriaria* Mayr, 1872, is also generated and compared with the closely related species, *C. modiglianii* Emery, 1900. Our analysis included the 3' region of COI region.

In the present paper, based on our examination of type materials of almost all of the forms under consideration

and nontype specimens including nest series samples, we revise their names and solve synonymy of taxa related to the species. In addition, this study redescibes the queen and male castes of the species and then contributes toward the taxonomy on sexual forms of *Crematogaster* ants in species-level identification. The sequence data provide additional information for the species.

2. Materials and Methods

2.1. Sources of Material. Type and nontype specimens were examined and/or deposited in the collections listed below. Codes for public institutions generally follow those in [12].

KUEC: Institute of Tropical Agriculture, Kyushu University, Fukuoka, Japan.

MHNG: Musée d'Histoire Naturelle, Geneva, Switzerland.

NHMW: Naturhistorisches Museum, Wien, Austria.

2.2. Observation. Most observations were made at various magnifications on a Leica M205C stereomicroscope. Images were taken using a Canon EOS 50D with Canon MP-E 65 mm 1–5x Macro Lens and then processed using Combine ZM.

2.3. Measurements and Indices. Measurements were made under a Leica M205C stereomicroscope using micrometers. All measurements are expressed in millimeters, recorded to the second decimal place. The measurements for petiole and postpetiole follow [13] and those for queen and male specific measurements follow [8].

Data were plotted on log-log scales and regression lines calculated for workers using R version 2.14.2.

Head width (HW): maximum width of head in full-face view, excluding the eyes, in the worker and queen castes, but measured including the eyes in the male caste.

Head Length (HL): perpendicular distance from vertex margin to line tangent anteriormost projections of clypeus in full-face view.

Cephalic Index (CI): $HW/HL \times 100$.

Scape length (SL): length of the first antennal segment, excluding the neck and basal condyle.

Scape Index (SI): $SL/HW \times 100$.

Eye Length (EL): maximum length of the compound eye.

Pronotal Width (PW): maximum width of the pronotum in dorsal view in the worker caste.

Weber's length of the mesosoma (WL): diagonal length, measured in lateral view from the anterior margin of the pronotum (excluding the collar) to the posterior extremity of the propodeal lobe.

Propodeal Spine Length (PSL): measured from tip of propodeal spine to closest point on outer rim of propodeal spiracle in the worker caste but measured

from tip of propodeal spine to propodeal declivity in lateral view in the queen caste.

Petiole Length (PtL): length of the petiole in lateral view.

Petiole Width (PtW): maximum width of petiole in dorsal view.

Petiole Height (PtH): height of the petiole in lateral view.

Postpetiole Length (PpL): length of the postpetiole in lateral view (see [13, Figure 2]).

Postpetiole Width (PpW): maximum width of postpetiole in dorsal view, excluding the helcium.

Petiole Height Index (PtHI): $PtH/PtL \times 100$.

Petiole Width Index (PtWI): $PtW/PtL \times 100$.

Postpetiole Width Index (PpWI): $PpW/PpL \times 100$.

Waist Index (WI): $PpW/PtW \times 100$.

Queen and male specific measurements [8] are as follows:

Mesonotal width (MSNW): maximum width of mesonotum in dorsal view.

Mesonotal length (MSNL): maximum length of mesonotum in dorsal view.

Mesonotal Index (MSNI): $MSNW/MSNL \times 100$.

2.4. Genetic Analysis. Genomic DNA was extracted from tissues rich in mitochondria (e.g., legs) using a DNeasy Blood & Tissue kit (Qiagen, Maryland, USA). A 711 bp region of the mitochondrial genome, 3' region of the cytochrome oxidase I (COI), was amplified via the polymerase chain reaction (PCR) using primers "Jerry" 5'-CAACATTATTTTGGATTTTGG-3' and "Pat" 5'-TCCAATGCACTAATCTGCCATATTA-3' [14]. Reactions were carried out at 10 μ L volumes in a PCR Thermal Cycler MP (TaKaRa Bio Inc.) under the following conditions: a first cycle of 94°C for 1 min, followed by 5 cycles of 94°C for 1 min, annealing at 48°C for 90 s, and 72°C for 90 s, and then 30 cycles of 94°C for 1 min, annealing at 51°C for 90 s, and finally 72°C for 90 s for the COI.

PCR products were visualized on a 1% agarose E-Gel 96-well system (Invitrogen) and then purified with 1.0 μ L of ExoSAP-IT (GE Healthcare Life Sciences). All products were sequenced in both directions using BigDye Terminator v3.1 (Applied Biosystems) on an ABI 3100 Avant DNA Sequencer (Applied Biosystems) at the Faculty of Science, Kyushu University, Fukuoka. It is noted that these sequences are not DNA barcoding region. DNA sequence data for one individual of *Crematogaster coriaria* Mayr, 1872, and closely related species, *C. modiglianii* Emery, 1900, were thus generated and deposited at DNA Data Base of Japan, DDBJ (with accession numbers: LC068833, for *C. coriaria* Mayr, 1872, and LC068834 for *C. modiglianii* Emery, 1900). *Crematogaster coriaria* Mayr, 1872, was sequenced from one nest series (SH10-Mal-85) from Ulu Gombak, Malaysia (Peninsular Malaysia), and *C. modiglianii* Emery, 1900, from

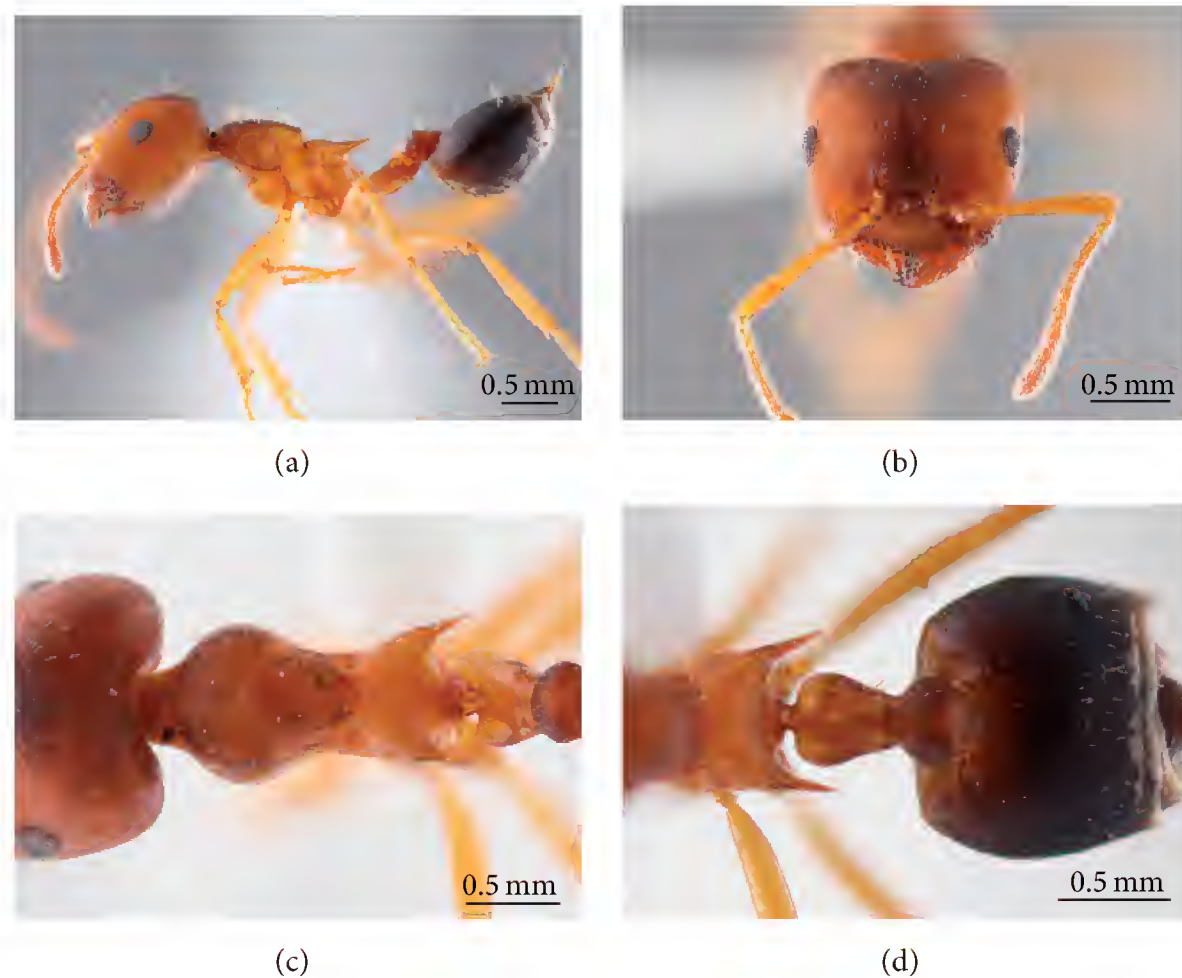


FIGURE 1: *Crematogaster coriaria* Mayr, 1872, worker. (a)–(d) Large worker (HW 1.39; WL 1.52) (Ulu Gombak, Selangor, Malaysia): (a) body in lateral view; (b) full-face view of head; (c) dorsal view of mesosoma; (d) petiole and postpetiole in dorsal view.

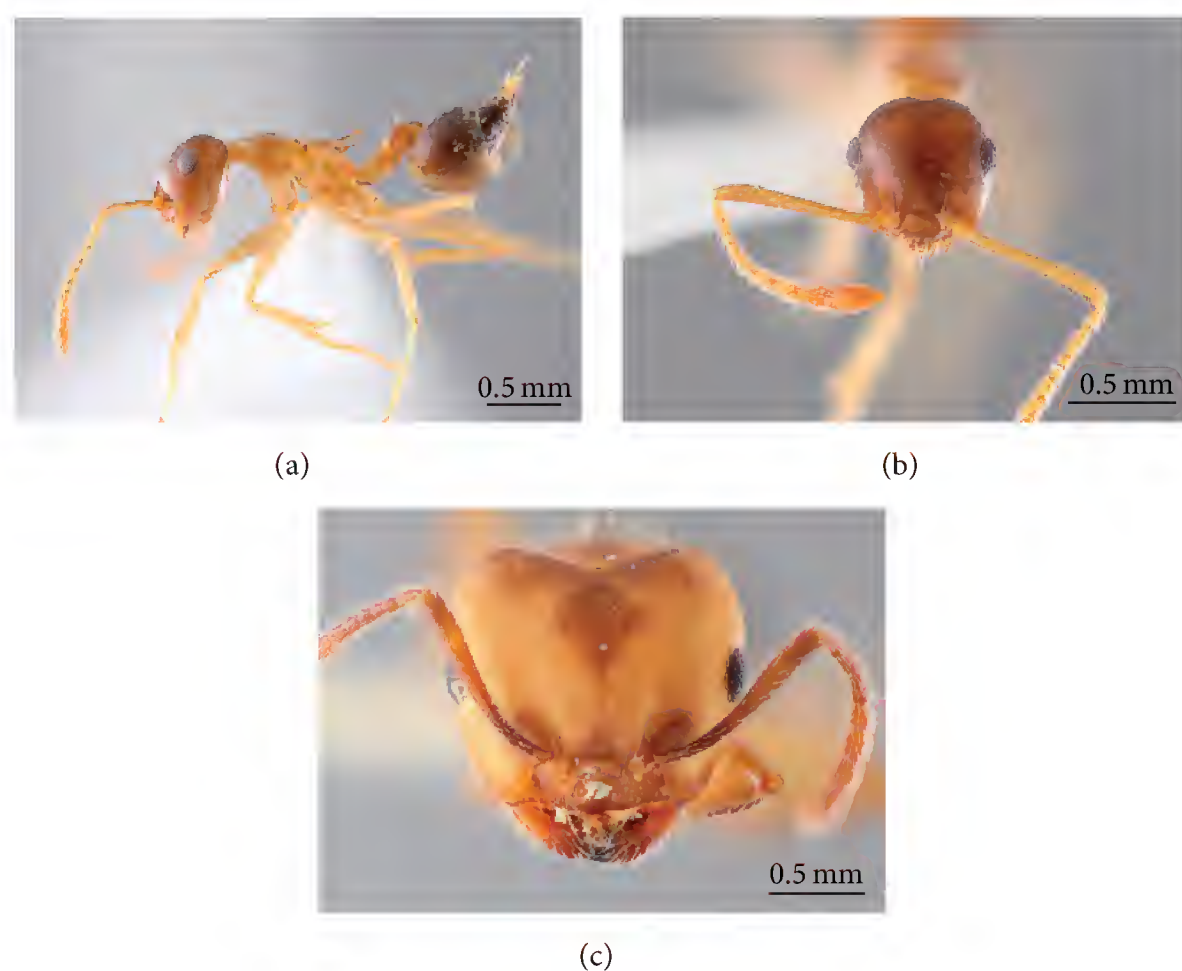


FIGURE 2: *Crematogaster coriaria* Mayr, 1872, worker. (a)–(c) Small worker (HW 0.64; WL 0.88) (Ulu Gombak, Selangor, Malaysia): (a) body in lateral view; (b) full-face view of head; (c) large worker having ocelli (HW 1.63; WL 1.58) (Cikaniki, Halimun, Java, Indonesia).

one nest series (SH10-Mal-18) from Ulu Gombak, Malaysia (Peninsular Malaysia). Contigs were assembled using Vector NTI Advance TM ver. 11 (Invitrogen Corp.) and subsequently aligned by eye. Genetic distances were estimated using the Kimura-2-parameter [15] distances with MEGA 5 [16].

3. Results

3.1. Taxonomy

Crematogaster coriaria Mayr, 1872 (see Figures 1–4). *Crematogaster coriaria* Mayr, 1872: 154 [1]. LECTOTYPE worker (by present designation) and three paralectotype workers from

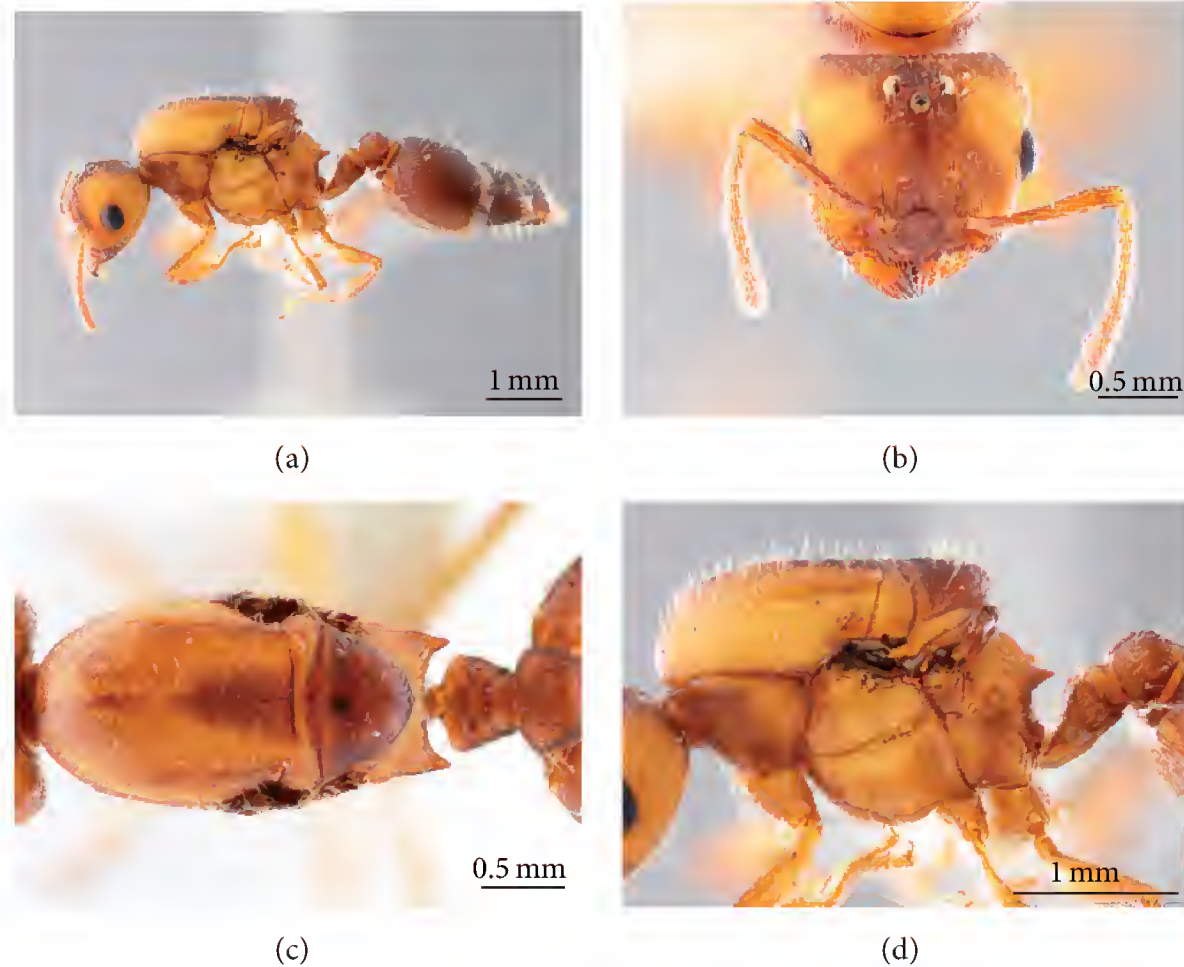


FIGURE 3: *Crematogaster coriaria* Mayr, 1872, queen: (a) body in lateral view; (b) full-face view of head; (c) dorsal view of mesosoma; (d) lateral view of mesosoma, petiole, and postpetiole.

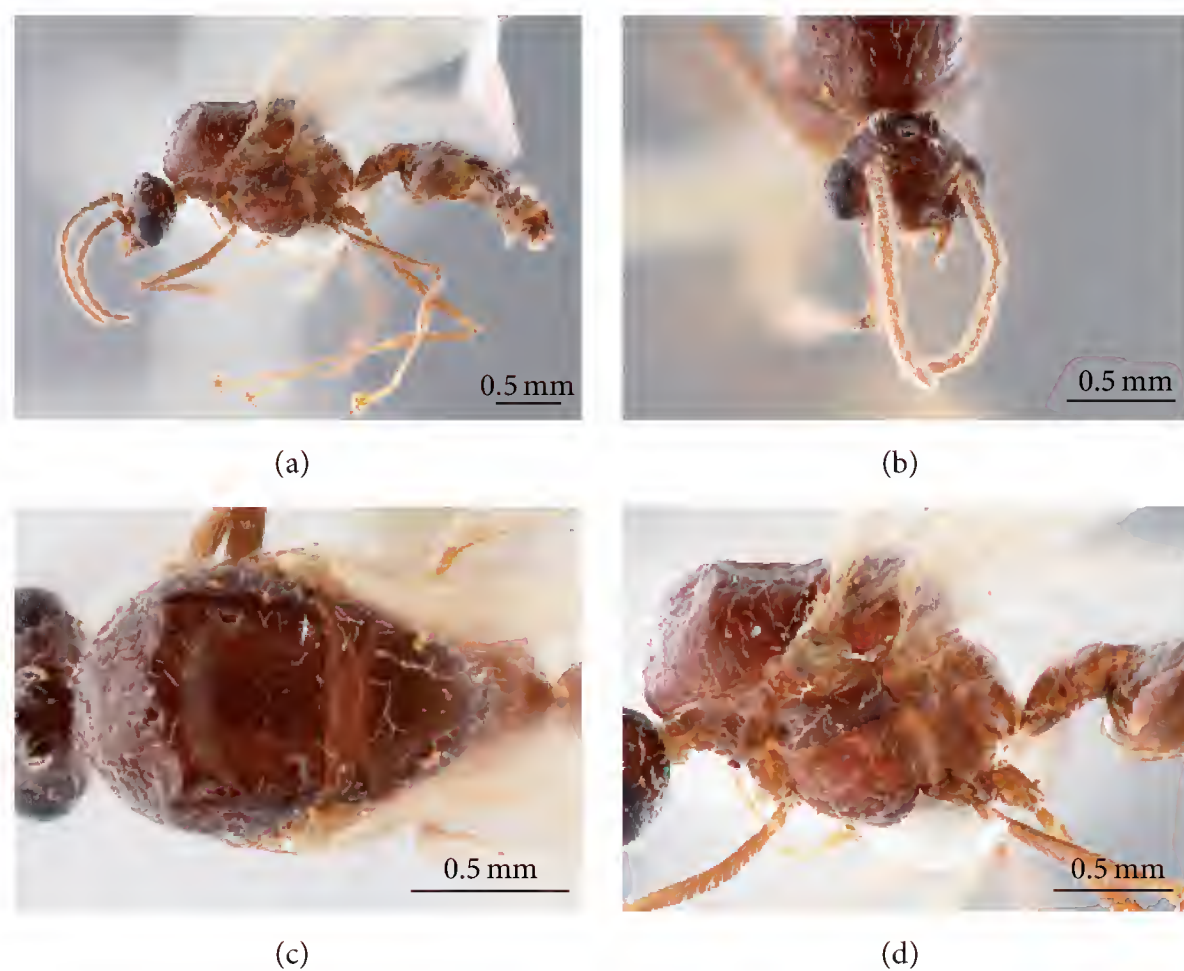


FIGURE 4: *Crematogaster coriaria*, Mayr, 1872, male: (a) body in lateral view; (b) full-face view of head; (c) dorsal view of mesosoma; (d) lateral view of mesosoma, petiole, and postpetiole.

MALAYSIA: Borneo, Sarawak, 1865-66 (G. Doria) (NHMW, MHNG) (examined). Combination in *C. (Paracrema)* by Emery, 1922: 155 [17]; in *C. (Crematogaster)* by Blaimer, 2012: 55 [3].

Crematogaster coriaria subsp. *fraudatrix* Forel, 1911: 376 [2]. LECTOTYPE worker (by present designation) from INDONESIA: Sumatra. (*Bluntschli*) (MHNG) (examined).

Combination in *C. (Paracrema)* by Emery, 1922: 155 [17]. *syn. nov.*

Crematogaster spengeli Forel, 1912: 56 [18]. LECTOTYPE worker (by present designation) from INDONESIA: Sumatra, Indrapura. (*Tritschler*). (MHNG) (examined). Combination in *C. (Paracrema)* by Santschi, 1918: 182 [19]; in *C. (Crematogaster)* by Blaimer, 2012: 55 [3]. *syn. nov.*

Crematogaster spengeli r. *taipingensis* Forel, 1913: 75, figure V, [20]. LECTOTYPE worker (top specimen of three on one pin) (by present designation) and two paralectotype workers from MALAYSIA: Taiping, Maxwell Hill. (MHNG) and three paralectotype workers from MALAYSIA: Bandar Baru (MHNG) (examined). Combination in *C. (Paracrema)* by Emery, 1922: 156 [17]. *syn. nov.*

Worker Measurements and Indices. HW 0.64–1.63; HL 0.68–1.49; CI 93–110; SL 0.81–1.19; SI 73–128; EL 0.18–0.30; PW 0.43–0.88; WL 0.88–1.58; PSL 0.19–0.37; PtL 0.24–0.46; PtW 0.25–0.52; PtH 0.16–0.32; PpL 0.14–0.32; PpW 0.25–0.48; PtHI 60–90; PtWI 91–131; PpWI 144–200; WI 88–107 (fifty-three workers measured).

General Description of Worker. Workers with pronounced size polymorphism.

Head subquadrate in full-face view, with weakly concave posterior margin, angular posterior corners, and subparallel sides; posterior portion developed in large workers (Figures 1(b) and 2(c)). Ocelli rarely developed in large workers (Figure 2(c)). Occipital carinae developed. Mandible with four teeth in small workers and five in large workers, apical and subapical teeth large. Anterior margin of clypeus convex with slightly impressed median portion; anterolateral margins of clypeus protruded anteriorly; posterior margin of clypeus rounded between frontal lobes. Frontal carinae almost parallel. Antennae 11 segmented; relative scape length variable in several worker size; antennal club 4-segmented. Scape exceeding posterior corner of head. Compound eyes distinctly projecting beyond lateral margins of head in full-face view in small workers (Figure 2(b)), but not distinctly projecting in large workers (Figures 1(b) and 2(c)).

Pronotum and mesonotum almost fused without defined suture, rarely promesonotal suture present in large workers. Posterior margins of mesonotum forming triangle-shaped process in lateral view in small workers (Figure 2(a)), but mesonotal dorsum forming flat outline in large workers (Figure 1(a)). Mesothoracic spiracle reduced to form small pit dorsoventrally. Metapleural gland opening slit-shaped. Propodeal spiracle elliptical, situated at posterolateral corners, touching metapleural gland bulla. Metanotal groove straight in dorsal view, deep and forming concave region between mesonotum and propodeum. In dorsal view, feeble longitudinal rugulae connecting between mesonotum and propodeum; the boundary distinct. Propodeal spines developed long.

Petiole scoop shaped, but flattened with convex sides or broader anteriorly, longer than broad; spiracle situated anteriorly midportion between dorsal and ventral margin of petiole in lateral view, directed laterally. Postpetiole without distinctly longitudinal median sulcus, but bilobed behind in large workers; spiracle situated anteriorly on lateral surface.

Posterior half of head sculptured without rugulae; gena with rugulae on surrounding antennal sockets. Clypeus with abundant longitudinal rugulae in large workers (Figures 1(b) and 2(c)), but weakly punctuated in small workers (Figure 2(b)). Promesonotum weakly punctuated. Lateral surface of pronotum smooth and shining. Mesopleuron

densely sculptured. Anterodorsal surface of propodeum generally sculptured. Lateral surface of propodeum smooth and shining. Dorsal surface of petiole smooth and shining. Lateral surface of petiole weakly punctuated. Dorsal surface of postpetiole sculptured. Lateral surface of postpetiole generally smooth and shining.

Standing pilosity sparse. Dorsal face of head with some pairs of long and erect setae. Clypeus with some pairs of erect setae. Anterior clypeal margin with two pairs of long setae mixed with short setae laterally. Mesonotal dorsum with sparse erect setae. Petiole with one pair of suberect setae posteriorly. Postpetiole with one pair of suberect setae posteriorly. Fourth abdominal tergite with appressed setae and sparse erect setae.

Body color yellow-brown.

Queen Measurements and Indices. HW 1.54–1.59; HL 1.39–1.42; CI 111–112; SL 1.09–1.16; SI 71–73; EL 0.40–0.43; MSNW 1.38–1.39; MSNL 2.38–2.48; MSNI 56–58; WL 2.77–2.78; PSL 0.19–0.20; PtL 0.55–0.56; PtW 0.65–0.66; PtH 0.47–0.49; PpL 0.38–0.43; PpW 0.65–0.69; PtHI 85–88; PtWI 118; PpWI 160–171; WI 100–105 (two queens measured).

Description of Queen. With worker character conditions, except as follows.

Masticatory margin of mandibles with five teeth. Antennal scapes reaching posterior margin of head.

Mesosoma slender (MSNI 56–58, WL 2.77–2.78); mesoscutum in dorsal view about 2 times longer than broad. Propodeal spines short and stout. Petiolar spiracles situated below lateral tubercles. Subpetiolar process absent. Postpetiolar spiracles situated at lateral tubercles.

Dorsal surface of head with longitudinal rugulae, but posterior half weakly striated (Figure 3(b)). Mandibles generally smooth. Clypeus with abundant longitudinal rugulae. Mesonotum weakly shining, but weakly sculptured (Figure 3(c)). Lateral surface of pronotum and mesopleuron with feeble longitudinal rugulae (Figure 3(d)). Dorsal surface of propodeum with longitudinal rugulae (Figure 3(c)). Lateral surface of propodeum with distinct longitudinal rugulae (Figure 3(d)). Dorsal surface of petiole weakly sculptured (Figure 3(c)). Lateral surface of petiole sculptured (Figure 3(d)). Dorsal and lateral surface of postpetiole sculptured (Figure 3(c)).

Dorsal surface of head with sparse erect setae and abundant appressed setae. Anterior clypeal margin with one to two pairs of long setae mixed with short setae laterally. Scape with decumbent to appressed long setae. Mesonotal dorsum with erect setae sparsely. Fourth abdominal tergite with erect setae and appressed setae sparsely.

Male Measurements and Indices. HW 0.80; HL 0.58; CI 137; SL 0.15; SI 19; EL 0.29; MSNW 0.89; MSNL 1.46; MSNI 61.2; WL 1.54; PSL 0; PtL 0.27; PtW 0.28; PtH 0.23; PpL 0.18; PpW 0.32; PtHI 84; PtWI 101; PpWI 178; WI 116 (one male measured).

Description of Male. Mandibles very short, masticatory margin with two teeth, one large apical, but a blunt second lobe-like. Clypeus weakly convex with almost straight median.

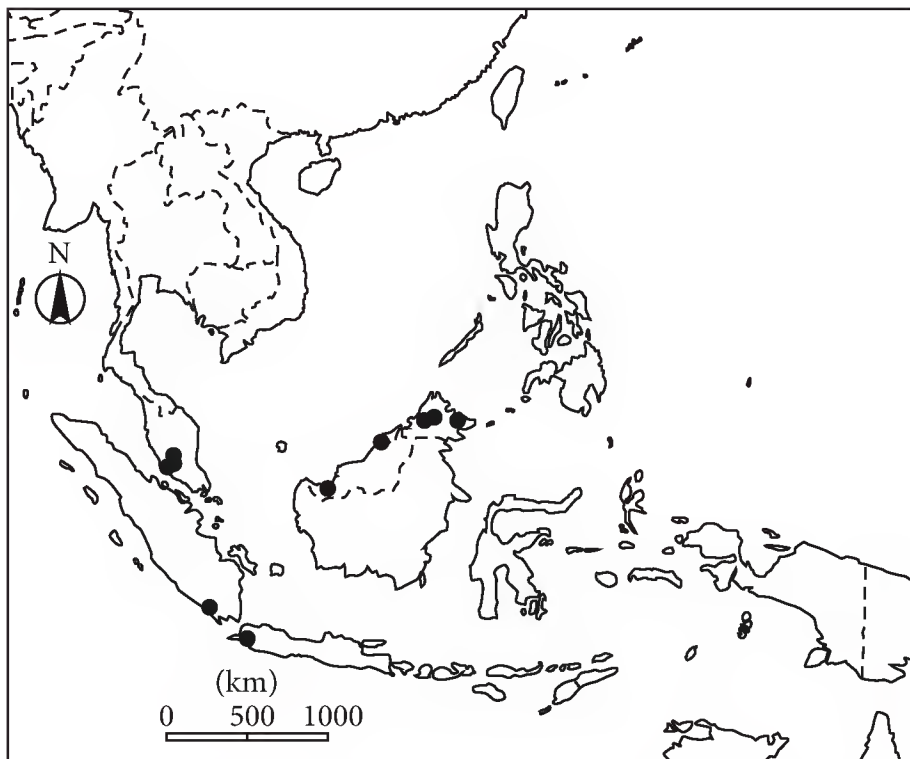


FIGURE 5: Distribution map of *Crematogaster coriaria* Mayr, 1872, specimens examined during this study.

Compound eyes large and protruding, midline of eyes situated well below midline of head. Antennae 12-segmented; flagellar segment longer than broad in apical to second basal segments (antennal segments III to XII). Head much wider than long (CI 137). In full-face view ocellar triangle situated at posterior head margin and elevated with respect to rest of face. Occipital carinae distinct.

Mesonotum in dorsal view longer than broad (MSNI 61.2); scutellum in dorsal view broadly tapering from anterior to posterior end, dorsoposteriorly rounded. Metanotum not protruding from below scutellum. Dorsal face of propodeum almost as long as posterior face. Propodeal spines absent. Petiole in dorsal view oval, weakly convex laterally, in lateral view anteriorly tapering. Subpetiolar process absent. Post-petiole almost round, but weakly bilobed posteriorly, lacking median impression.

Head sculpture shiny to rugulose (Figure 4(b)). Lower genal area surrounding antennal sockets with distinct rugulae. Mandibles striated with longitudinal rugulae. Clypeus shiny, but weakly sculptured. Mesoscutum generally smooth and shining, but anterior face with feeble rugulae (Figure 4(c)). Mesopleuron generally smooth and shining (Figure 4(d)).

Dorsal surface of head with suberect setae sparsely. Mesosoma with decumbent setae sparsely; propodeum with erect setae sparsely. Petiole and postpetiole with erect to decumbent setae sparsely. Fourth abdominal tergite with suberect to decumbent setae sparsely.

Color brown.

Distribution and Biology. This species is distributed in Malaysia (Peninsula, Borneo) and Indonesia (Sumatra, Java) (Figure 5). This species inhabits well-developed forests and nests in the dead woods.

Remarks. This species is similar to *C. modiglianii* Emery, 1900, but can be distinguished by the rugulae surrounding

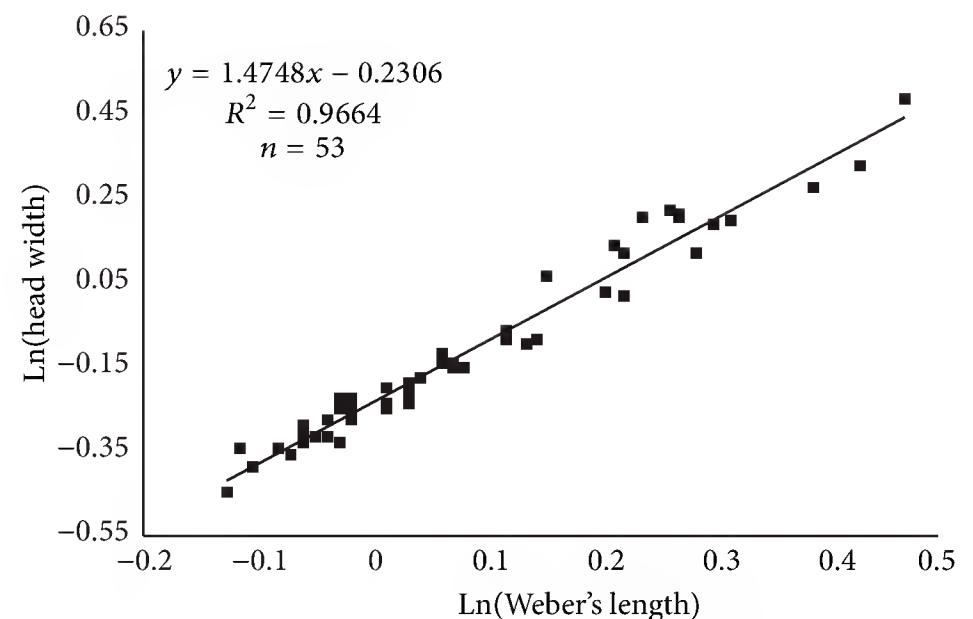


FIGURE 6: Relation between natural logarithms of Weber's length (WL) and head width (HW) in workers ($n = 53$) of *C. coriaria* Mayr, 1872.

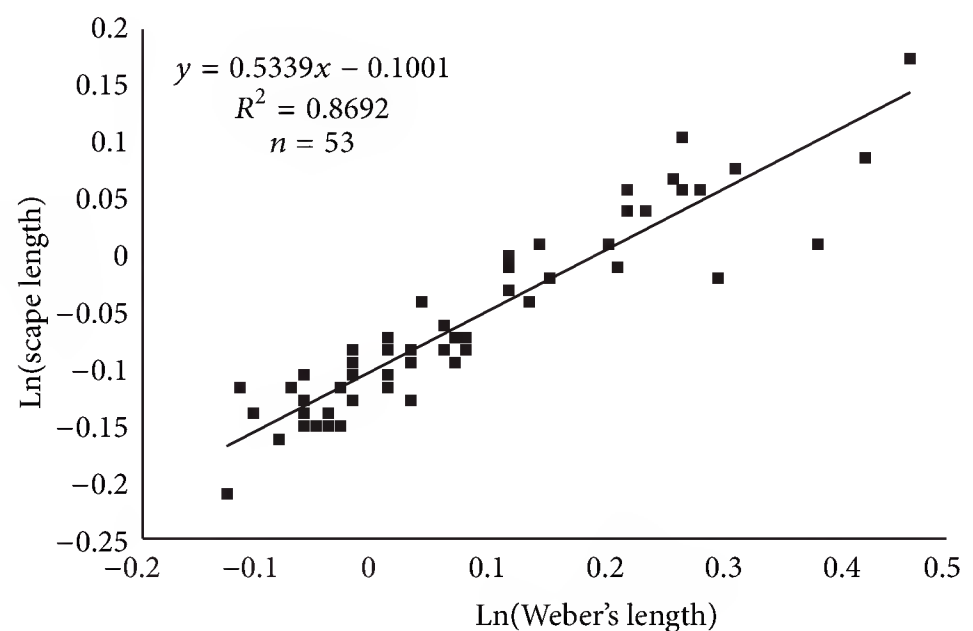


FIGURE 7: Relation between natural logarithms of Weber's length (WL) and scape length (SL) in workers ($n = 53$) of *C. coriaria* Mayr, 1872.

antennal sockets, mesonotal dorsum without a ridge on the median in the worker caste. *Crematogaster coriaria* Mayr, 1872, and *C. modiglianii* Emery, 1900, are geographically sympatric in the Peninsular Malaysia, Borneo, and Sumatra [21]. The COI divergence between *C. coriaria* Mayr, 1872 (Ulu Gombak specimen), and *C. modiglianii* Emery, 1900 (Ulu Gombak specimen), was relatively high (17.4% K2P distance), suggesting that the two taxa are distinct species.

The relation between Weber's length and head width showed a monophasic allometric growth (Figure 6). The slope of the regression line was greater than 1 ($t = 35.4$, $p < 0.01$), suggesting that large workers had proportionally wider head capsules than small workers. The relation between Weber's length and scape length also showed a monophasic allometric growth (Figure 7). The slope of regression line was lower than 1 ($t = 17.6$, $p < 0.01$), suggesting that large workers had proportionally shorter antennal scapes than small workers.

Specimens Examined. MALAYSIA: 1 worker, Mager Trail, Fraser's Hill ($3^{\circ}42'N$, $101^{\circ}44'E$), 15. iii. 2005 (2005c39)

(*S. Hosoiishi*) (KUEC); 1 worker, Bunga Buah, ca 1000 m alt., nr Genting H. L., Malay Pen. (3°25'N, 101°47'E), 6. vii. 1999 (*Sk. Yamane*) (KUEC); 9 workers, 1 male, Bunga Buah, ca. 1000 m alt., nr Genting H. L., Malay Pen. (3°25'N, 101°47'E), 6. vii. 1999 (MA99-SKY-15) (*Sk. Yamane*) (KUEC); 3 workers, Ulu Gombak, Selangor (3°19'N, 101°45'E), 2. xii. 2005 (SH05-Mal-13) (*S. Hosoiishi*) (KUEC); 8 workers, Ulu Gombak, Selangor (3°25'N, 101°47'E), 17. iii. 2009 (SH09-Mal-143) (*S. Hosoiishi*) (KUEC); 3 workers, Poring (600–700 m alt.), Sabah, Borneo, E. Malaysia (6°02'N, 116°42'E), 8. i. 1998 (*F. Yamane*) (KUEC); 8 workers, 2 queens, Danum Valley, Sabah, Borneo, E. Malaysia (4°33'N, 117°08'E), 1. v. 2000 (CB-00-15) (*C. Bruhl*) (KUEC); 1 worker, Danum Valley, Sabah, Borneo, E. Malaysia (4°33'N, 117°08'E), 1. v. 2000 (*C. Bruhl*) (KUEC); 13 workers, Mahua Waterfall, Crocker Range, Sabah (5°45'N, 116°30'E), 4. xi. 2000 (*B. T. Viet*) (KUEC); 2 workers, 8 ha plot, Tower Reg., Lambir, Miri, Sarawak, E. Malaysia (4°11'N, 114°02'E), 27. viii. 1994 (*T. Itioka & T. Yumoto*) (KUEC); 2 workers, 8 ha plot, Tower Reg., Lambir, Miri, Sarawak, E. Malaysia (4°11'N, 114°02'E), 26. viii. 1994 (*T. Itioka & T. Yumoto*) (KUEC); INDONESIA: 8 workers, around Cikaniki (ca. 1050 m alt.), G. Halimun NP, E. Java (6°43'S, 106°27'E), 14. viii. 2002 (JV02-SKY-07) (*Sk. Yamane*) (KUEC); 4 workers, Cikaniki (1050–1250 m alt.), G. Halimun, W. Java (6°43'S, 106°27'E), 25. i. 2004 (JV04-SKY-25) (*Sk. Yamane*) (KUEC); 2 workers, Bodong Jaya, Sumberjaya, Lampung Barat, S. Sumatra (5°10'S, 104°07'E), 18. ix. 2007 (SU07-SKY-200: secondary forest) (*Sk. Yamane*) (KUEC); 6 workers, Bodong Jaya, Sumberjaya, Lampung Barat, S. Sumatra (5°10'S, 104°07'E), 16. ix. 2007 (SU07-SKY-154) (*Sk. Yamane*) (KUEC); 4 workers, Bodong Jaya, Sumberjaya, Lampung Barat, S. Sumatra (5°10'S, 104°07'E), 16. ix. 2007 (*Gede*) (KUEC); 2 workers, Bodong Jaya, Sumberjaya, Lampung Barat, S. Sumatra (5°10'S, 104°07'E), 17. ix. 2007 (*Sk. Yamane*) (KUEC).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Body Size of the Monomorphic Ant *Lasius niger*: Young Colonies along a Metal Pollution Gradient

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Metal pollution may cause the decrease in the individual body size. In ants, the morphological diversity within and between colonies may be much higher than that considered before, even in monomorphic ants. In this study we measured the body size, expressed as head width, of *Lasius niger* workers collected from 44 young colonies in their ergonomic stage along a well-known gradient exhibiting chronic metal pollution. We calculated statistics describing the body size distribution curve, namely, average, median, data range, skewness, and kurtosis. None of these statistics correlated with the pollution level. Contrary to our previous study performed on mature colonies, workers from young colonies do not display pollution-related morphological changes. The results stress the importance of developmental stage of colony on diversifying body size of the worker cast, in monomorphic ants living in metal-polluted areas.

1. Introduction

Some social insects have evolved morphologically distinct subcastes of workers, dedicated to different tasks in the colony. However, most ant species and genera maintain a single behaviorally flexible worker cast [1]. Despite being monomorphic, considerable inter- and intracolony worker size variation may exist [2, 3]. Although such a variation was generally considered to be too small to be relevant for task allocation, the recent study has effectively challenged this claim. Workers of the monomorphic species *Temnothorax rugatulus* foraging outside the nest might be bigger than intranidal workers [4]. Therefore, monomorphism does not necessarily mean the absence of body size-related division of labour. The question can also be asked as to what extent the body size of monomorphic ants can be modified in polluted and stressed environments. Field studies as well as laboratory experiments showed that in many invertebrate species individual body size may decrease along the metal pollution gradient [5–7]. Significant decreases in body size have also been observed along urbanization gradients [8, 9]. However, as many other authors indicated, the relationship between body size and urbanization or metal pollution

pressure might be much more complex and cannot always be described as simple increase or decrease [10–12].

In our previous studies we analyzed the body size of the monomorphic ant *Lasius niger* (L., 1758) along a well-known gradient of metal pollution [13]. We tested body size under the expectation that it will decrease along the increasing metal pollution gradient as a result of toxicity and/or food limitation. We found that the average body size was unrelated to pollution, but the frequency of relatively small ants increased with the pollution level. Thus, the shape of body size distributions seems to respond to environmental stress, although the investigated ant is considered monomorphic [14]. We found similar increase of relatively small ants without a change in the median or average size in related species *Lasius flavus* (Grześ, Okrutniak unpublished). We proposed a hypothesis that the observed change might be underlined by energetic constraints in the colony. The study on *Lasius niger* was undertaken on mature colonies, at the reproductive stage, namely, the stage of intensive sexual cast production, that is energetically costly to breed [15]. Therefore, the question appears to be whether the observed change depends on the developmental stage of the colony.

In the present study we investigated 44 young nests (not producing sexual casts) of *Lasius niger* located along a well-known pollution gradient, which was the same as the one used in the previous study. The body size (expressed as head width) was measured. We compared the statistics describing the central tendency and distribution shape along the pollution gradient.

2. Materials and Methods

2.1. Study Species and Study Area. The black garden ant (*Lasius niger*) is one of the most common species in the Palearctic area [14]. Our previous study revealed that *L. niger* is the most abundant ant, compared to other species inhabiting the investigated area [16]. The species inhabit various anthropogenic environments like farmlands, pastures, ruderal places, or green spaces in urban areas. *L. niger* is strictly monogynous and monomorphic, building mineral nest mounds containing from a hundred to more than ten thousand workers. The species of interest is both aphidicolous and carnivorous [14].

The study area is located in the vicinity of Olkusz in Southern Poland. Metal concentrations in the humus layer at the most polluted sites in this region exceed 9600 mg/kg for zinc, 1500 mg/kg for lead, and 80 mg/kg for cadmium [17]. Previous studies performed in the area showed that metal concentrations in the soil are highly correlated with each other [10, 18]. Fifteen study sites were established along the pollution gradient covering abandoned fields and fresh meadows. The transect extended from 0.7 to 35 km from the pollution source. Metal concentrations at the sites decreased with an increasing distance to the smelter, approaching background levels at the sites further than 25 km from the smelter. The study sites were represented by abandoned fields (11 sites), mown meadows (1 site), and industrial wastelands (3 sites).

2.2. Pollution Level. In order to provide a biologically realistic measure of the pollution level of each site, we analyzed Zn and Cd concentrations in a random sample of invertebrates collected at each site in a preliminary study in the summer of 2011. Zn concentration in invertebrates correlated positively with both the Cd concentration in invertebrates and the Zn total soil concentrations. The lowest and highest Zn concentrations in invertebrates accounted for 134 and 1,545 mg/kg dry weight (d.w.). In turn, the lowest and highest Cd concentrations in invertebrates accounted for 3.98 and 39.70 mg/kg d.w. The detailed Zn and Cd concentrations in invertebrates and in the soil samples as well as the botanical description of the study sites are reported in [13] (see sites S1–S3, S5, S6, S8, S10–S12, and S14–S19). Zn concentration in invertebrates was used as an indicator of pollution level.

2.3. Morphological Measurements. The species identification followed [14]. In each of the 15 study sites, 3 young colonies of *L. niger* were selected ($15 \times 3 = 45$). One colony was excluded from the final analysis due to the mistake during sampling. All colonies were checked for the absence of sexual

casts. A random sample of about 30 ± 3 workers from each nest was collected using exhaustor and stored in -5°C till being measured. The body size of ants was expressed as the maximum head width above the eyes (HW) [14]. All measurements of head widths were performed under 50x magnification using a metallographic microscope Met-153 (Motic, China) connected to a digital camera (Huvitz, Korea). The head widths were measured to the nearest 0.00001 mm based on digital photos using Panasis, ver. 2.4.2, Huvitz. In order to check the accuracy of the measurements 30 ants were measured twice. The results of the first and the second set of measurements were correlated. High correlation coefficient ($r = 0.90$, $P < 0.0001$) indicated high repeatability of the measurements. In *L. niger* ants, the nest volume grows almost proportionally to the colony size [19]; therefore, the nest basal area diameter was used as an estimator of the colony size. In all colonies used in this study, the mound diameter ranged from 10 to 47 cm (median size accounted for 19.0 cm).

2.4. Statistical Procedures. To test the relationships between the pollution level and body size of *L. niger*, the descriptive statistics of head width, including mean, median, and range, as well as kurtosis and skewness, were calculated. Each of these statistics was calculated on each colony and was correlated separately with the pollution levels of the sites (Zn concentration in the invertebrates). The significance of each relationship was tested using a simple regression (Statgraphics Centurion XV) with a 95% confidence level followed by a check for normality of residuals. In ants the body size can be correlated with the size of colony [20–22]. Therefore, in order to eliminate the influence of the colony size prior to correlating with the pollution level, each statistic was first correlated with the colony size. The residuals were then regressed against the pollution level. Thus, when testing the relationship between head size and pollution, only the fraction of variance that was not explained by the colony size was used. As three ant nests were collected at each site, the values of residuals for each of the statistics were averaged to avoid a problem of pseudoreplication [23].

3. Results

The average head size ranged between 700 and 965 μm . The head size differs significantly between colonies (one-way ANOVA, $F = 35.52$, $P < 0.0001$; Figure 1). The size distribution in each colony did not depart significantly from the normal distribution as both st. skewness and st. kurtosis were within the range expected for normal distribution -2 to $+2$. No statistics (average, median, range, st. skewness, and st. kurtosis) correlated significantly with pollution of the site ($P > 0.05$ in each case, Table 1).

4. Discussion

The aim of this study was to test if the bias towards the higher frequency of small ants, detected in previous studies in mature colonies of *Lasius niger*, occurs also in the younger colonies of this species. Based on our study no significant

TABLE 1: The results of linear regression of statistics describing the body size distribution of *Lasius niger* workers versus pollution level. Five independent analyses were done. None of the statistics were significantly correlated with the metal pollution gradient.

Statistics	F	R^2	P	Interpretation
Average	0.91	0.065	0.56	Central tendency measurements are independent of the pollution levels of the sites.
Median	0.81	0.059	0.38	
Range	2.99	0.187	0.11	The range of dispersal of body size per site is not dependent on the pollution levels of the sites.
st. skewness	0.19	0.147	0.67	The distribution shape is independent of the pollution levels of the sites.
st. kurtosis	0.99	0.071	0.34	

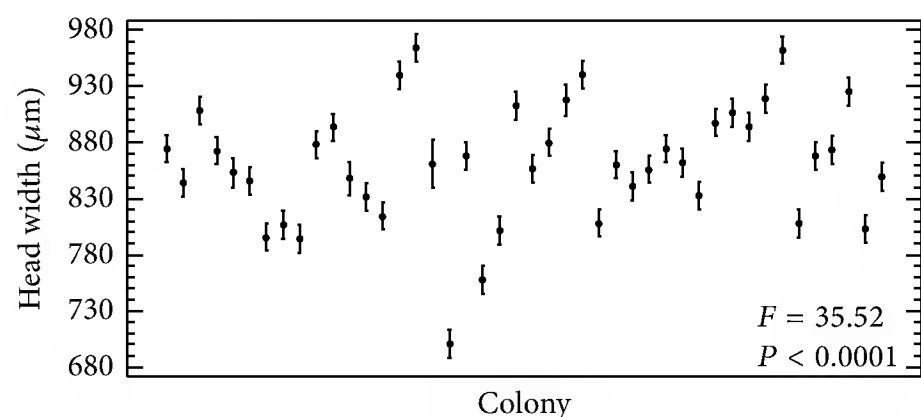


FIGURE 1: The results of one-way ANOVA of head size of the ant *Lasius niger* collected from 44 colonies. Points represent the mean value calculated per colony. Whiskers denote 95% intervals of LSD test. Since $P < 0.0001$, the colonies differ significantly in average head size of workers.

increase of the fraction of small ants was found along the pollution gradient. In turn, big and mature colonies were found previously to contain larger fraction of small workers along the increasing metal pollution gradient. Thus, the change in the shape of body size distribution in the investigated ant seems to be associated with the developmental stage of the colony. In both previous and present study we used the same species, study area, measurement procedures, and a similar number of colonies ($N_{\text{mature}} = 51$, $N_{\text{young}} = 44$) and identical number of individuals per colony ($N = 30$). Therefore, we believe that the comparison between mature and young colonies in the study should be mandatory.

Trace metal pollution is known to cause a range of toxic effects on invertebrates [24–27]. Considering the effects on morphology, metal pollution may induce morphological deformities [28], the increase in developmental instability [29, 30], and reduced body size [6, 31]. Generally, three mechanisms may underline the negative impact of metals on the body size/mass: (1) toxicity of metals based on the displacement of the essential metals from enzymes at active sites, resulting in loss of their function, (2) trade-offs between the cost of detoxication and energy allocation to growth and development [6, 32], and (3) limited food availability in contaminated areas [7]. In this study we conclude that *L. niger* may undergo certain morphological changes along the pollution gradient, but the change seems to be associated with mature colonies but not with young colonies. This observation might be explained in the light of the colony development and energy allocation within the colony.

In ants, three main phases of colony development over its lifespan can be distinguished [33]. The first phase called

“colony founding” is characterized by the absence of adult workers; the only adult present is the egg laying queen. The development of the colony at this stage is weakly dependent on external food resources but remains heavily reliant on the amount of fat accumulated in the queen. The moment of transition to the next stage of colony development, namely, the ergonomic stage, is eclosion of the first workers. At this stage the queen is focused exclusively on egg laying, while the workers are concerned with brood care. As a consequence, at this stage the energy investments in the colony are devoted to colony growth rather than reproduction or dispersal. When the number of adult workers approaches a certain level, the production of the virgin queens and males starts; thereby the colony enters the reproductive stage. Therefore, young colonies allocate their resources to rearing workers, thus facilitating colony growth, while mature colonies have to share the energy to be allocated between a new generation of workers and in sexual castes. Rearing males and females in mature colonies that are at the reproductive stage most likely needs more effort than simply rearing workers in the young colonies at the same reproductive stage. Tschinkel observed that workers of the *Pogonomyrmex* ants lose 20% fat during the male and female feeding phase that takes place in July [34]. Therefore, the increased fraction of small workers observed in mature colonies, but not in young colonies, might be an effect of energy limitation during intensive production of sexuals.

5. Conclusions

We did not find the pollution-related increase of the fraction of relatively small workers in the young colonies of *L. niger* to be similar to that found in the previous analogical research performed on the mature colonies. The results of this study suggest that body size in monomorphic ant *Lasius niger* may undergo shifts in the distribution of the body size as a result of the interaction of metal pollution and the stage of the colony development. Energetic constraints during the sexual casts rearing might explain the proved difference between young and mature colonies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effects of Pyriproxyfen on Life Table Indices of *Plutella xylostella* in Multigenerations

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The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a major pest of Brassicaceae family in Iran. This study investigated the sublethal effects of pyriproxyfen, a juvenile hormone analogue, on DBM, for two subsequent generations. The leaf dip bioassay method was adopted in conducting this experiment. Sublethal concentrations resulted in a significant decrease in fecundity, oviposition period, and pupal weight of parents and offspring generations. Also, the development time of DBM was prolonged after exposure to pyriproxyfen. The biological parameters such as net reproductive rate (R_0) and intrinsic (r_m) and finite (λ) rate of increase were lower than control in treatment groups, significantly. From our investigations, pyriproxyfen is a good choice for control of the diamondback moth population through continuous generations.

1. Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the most destructive insect pests of Brassicaceae worldwide. Its annual cost for control is estimated to be US \$1 billion [1, 2]. It is ranked among the most resistant species [3]. DBM feeds only on members of the Brassicaceae such as radish, turnips, kohlrabi cabbage, broccoli, Brussels sprouts, cabbage, mustard, Chinese cabbage, and rape. These plants are the most common vegetables in Asian diet [1, 4]. The high reproductive rate, rapid resistance development to insecticides, and absence of effective natural enemies, especially parasitoids, are majorly responsible for the increasing pest status of DBM in most counties [5, 6].

Over the last two decades, DBM has been a major pest of cruciferous crops in Tehran province and other areas of Iran [7]. Owing to its ability to develop resistance to many conventional insecticides, the use of new insecticides which have low effects on other nontarget organisms can be effective and helpful. The use of very low doses of insecticides with strong sublethal effects offers a promising and

environmentally friendly component to improve integrated pest management strategies [8].

Pesticides at sublethal concentrations have a strong impact on insects physiologically and behaviorally [9]. The study of the life table parameters of an organism (demographic toxicology) can be a good indicator for background toxicology [10]. Fenoxycarb and pyriproxyfen are aromatic nonterpenoid insecticides which can mimic the action of JHs in a number of physiological processes [11]. Pyriproxyfen with a low mammalian toxicity was first registered in Japan in 1991 for control of public health pest [12]. The sublethal concentrations of IGRs and other groups of insecticides have been shown to affect reproductive parameters. For example, according to Sial and Brunner [8], the adult weight of Obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), was lowered upon application of pyriproxyfen. Also, fluctuations (increasing and decreasing) in the developmental times of various insects have been reported after exposure to the insecticides [13–17]. In addition, the chemical and natural compounds may affect the insects' larval weight [17–20] and other reproductive parameters such as the pupal weight [21], hatchability [19],

TABLE 1: Toxicity of pyriproxyfen on the 3rd instar larvae of diamondback moth, *Plutella xylostella*.

	n^a	df	LC ₁₀ (g L ⁻¹) ^b	LC ₂₅ (g L ⁻¹) ^b	LC ₅₀ (g L ⁻¹) ^b	Slope $\pm$ SE	χ^2
Pyriproxyfen	259	5	0.610 (0.394–0.764)	0.848 (0.643–0.988)	1.223 (1.070–1.362)	4.24 $\pm$ 0.74	7.30

a: number of subjects.

b: 95% confidence limits in parenthesis.

fecundity [17, 22], preoviposition and postoviposition periods [23], and other reproductive parameters such as net reproductive rate (R_0) and intrinsic rate of increase (r_m) [24, 25].

In a previous report, contact toxicity (topical) of pyriproxyfen on DBM was studied by Alizadeh et al. [26]. In the current study, leaf dip method which is a combination of contact and oral toxicity has been adopted. This combined method of pyriproxyfen application is similar to its recorded application in field. Therefore, the current results can be more functional for DBM control. This study was conducted to evaluate the sublethal effects and toxic activity of pyriproxyfen on the demographic parameters of DBM, such as fecundity and pupal weight, developmental time, preoviposition, oviposition, and postoviposition periods, adult longevity, and total life span. The oviposition parameters included gross reproductive rate (GRR), net reproductive rate (R_0), intrinsic rate of increase (r_m), finite rate of increase (λ), mean generation time (T), doubling time (Dt), birth rate (b), and death rate (d).

2. Materials and Methods

2.1. Insect Rearing. The *P. xylostella* larva was collected from an insecticides-free field at Faculty of Agriculture at Tarbiat Modares University, Tehran, Iran, during the 2013 growing season. Leaves of cauliflower, *Brassica oleracea* (Brassicaceae), were used in rearing the larva. A 10% sugar solution was used in feeding the adult. Insect colonies was maintained at $25 \pm 1^\circ\text{C}$ and $65 \pm 5\%$ relative humidity (RH) under a 16L:8D cycle in a growth chamber. The bioassays were started after three generations.

2.2. Dose-Mortality Response and Sublethal Effects on Parent Generation. The bioassay tests were performed through leaf dip method [27]. This was performed by dipping circular cabbage leaf disks into various concentrations of insecticide (pyriproxyfen, Admiral 10 EC, Sumitomo Chemical, Japan) prepared with water containing 0.02% Tween-20 for 10 s, under laboratory condition. Water and Tween-20 were used for control. After drying at room temperature, the leaf disks were placed in plastic cups. Thereafter, 10 randomly selected 3rd instar larvae were placed on each leaf disk. The test was repeated in 4 cycles. The mortality counts were recorded and analyzed 96 h after treatment using probit analysis SAS version 9.1 [28].

To determine the effect of sublethal doses on biological parameters of parent generation, the cabbage leaf disks were dipped in sublethal solutions for 10 s. Whenever the leaves had dried, twenty-five 3rd instar larvae were put on the treated leaf disks, kept in plastic cups, and allowed to feed for 96 h. Each treatment was replicated eight times.

The circular leaf disks were treated as explained in the previous section while 0.02% Tween-20 in water was used as a control. To measure the fecundity and other demographic parameters, the surviving larvae were transferred onto fresh cabbage leaves and allowed to continue their development to pupation. On the 2nd day of pupal period, the pupae in both the parent and offspring generations were individually weighed. To determine the oviposition parameters, fifteen pairs (male and female) from each treatment were set up and introduced into a plastic cage ($8.5 \times 6.5 \times 4$ cm) containing fresh leaf disks necessary for mating and oviposition. The adults were fed on a 10% sugar solution. Daily collection and replacement of leaves by new ones were necessary to prevent starvation of larvae.

2.3. Effects of Sublethal Doses on Offspring Generation. To evaluate the effect of pyriproxyfen on the next generation, 100 eggs obtained from the parent adults of each treatment were individually placed in plastic cages. The larvae were fed on cabbage leaves. The developmental time and survival rates of various stages were recorded daily until death of all individuals. Oviposition parameters were recorded as above.

2.4. Data Analysis. To test for the sublethal effects of treatments on the demographic parameters of *P. xylostella*, a one-way analysis of variance was performed. A significant difference was necessitated for the mean to be separated using Tukey's Studentized Range Test (at $P < 0.05$). All statistical tests were performed in SAS version 9.1 [28]. Jackknife technique [29] which is similar to bootstrapping was used to estimate the sample mean and standard error of biological parameters.

3. Results

3.1. Toxicity of Tests. Treatment with pyriproxyfen caused a high level of toxicity on *P. xylostella* (Table 1). The calculated LC₅₀ of leaf dip bioassay on the third instar larvae of *P. xylostella* was 1.223 g L^{-1} . The values of LC₁₀ and LC₂₅ measured 96 h after treatments were 0.610 and 0.848 g L^{-1} , respectively.

3.2. Pupal Weight and Fecundity. Sublethal concentrations of pyriproxyfen had determined effects on pupal weight and fecundity of parent and offspring generations as shown in Table 2. A significant decrease in the fecundity of both parent and offspring generations (ANOVA; parent, $F(2, 42) = 44.27$, $P < 0.0001$; offspring, $F(2, 46) = 9.10$; $P = 0.0005$) was observed when pyriproxyfen treatment was applied. Also, the pupal weight was significantly affected by pyriproxyfen in

TABLE 2: Comparison of fecundity of *P. xylostella* treated with sublethal doses of pyriproxyfen and control (untreated group) in the parent and offspring (F1 generation).

Entries	Fecundity (egg/female)	
	Parent Mean $\pm$ SE [#]	Offspring Mean $\pm$ SE [#]
Control	169.40 $\pm$ 12.84 ^a	148.00 $\pm$ 12.22 ^a
LC ₁₀	57.40 $\pm$ 10.13 ^b	79.54 $\pm$ 13.49 ^b
LC ₂₅	32.86 $\pm$ 9.55 ^b	55.55 $\pm$ 27.29 ^b
<i>F</i>	44.27	9.10
<i>P</i>	<0.0001	0.0005
df _{t,e}	2, 42	2, 46

#: means marked with the same letters within a column are not significantly different (Tukey's test; $P < 0.05$).

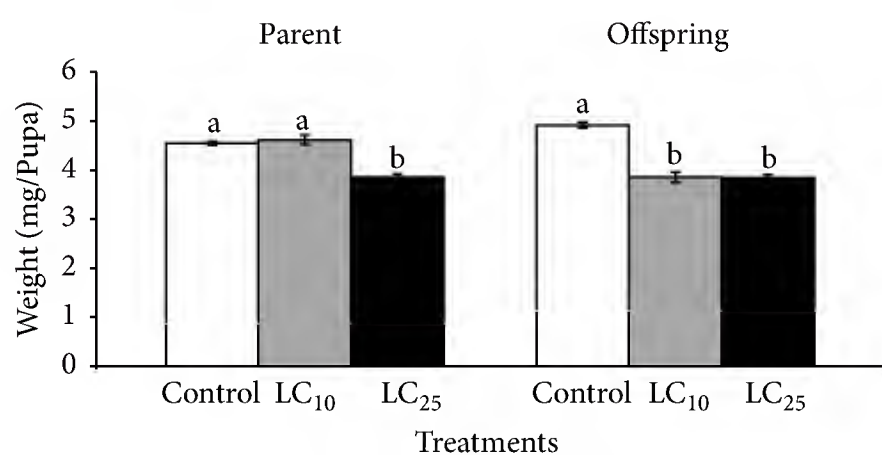


FIGURE 1: The effects of sublethal doses of pyriproxyfen on the pupal weight of *P. xylostella* in subsequent generations (parent: $F(2, 72) = 10.65$, $P < 0.0001$; offspring: $F(2, 72) = 10.67$, $P < 0.0001$).

parent and offspring generations (Figure 1) (ANOVA; parent, $F(2, 72) = 10.65$, $P < 0.0001$; offspring, $F(2, 72) = 10.67$, $P < 0.0001$).

3.3. Developmental Time and Adult Life Span. Pyriproxyfen significantly affected the developmental times of *P. xylostella* (Table 3). There was a significant extension in the development time of eggs in the treated groups compared to control (ANOVA; $F(2, 281) = 313.34$, $P < 0.0001$). Furthermore, the developmental time of 1st instar larvae was lower than control in LC₂₅ level (ANOVA; $F(2, 151) = 7.01$, $P = 0.0012$). Moreover, the developmental time of 2nd (ANOVA; $F(2, 122) = 3.83$, $P = 0.0244$), 3rd (ANOVA; $F(2, 114) = 6.03$, $P = 0.0032$), and 4th (ANOVA; $F(2, 101) = 37.65$, $P < 0.0001$) instar larvae were significantly shortened compared to control (Table 3).

The total developmental times were significantly lower at LC₁₀ and LC₂₅ levels than in the control group (ANOVA; $F(2, 99) = 22.29$, $P < 0.0001$). Unlike in the prepupal stage, the pyriproxyfen treatment caused a significant decrease in the pupal developmental time (ANOVA; prepupa, $F(2, 99) = 1.04$, $P = 0.3559$; pupa, $F(2, 94) = 6.69$, $P = 0.0019$). Preadult developmental time was significantly increased in the sublethal groups (ANOVA; $F(2, 94) = 52.37$, $P < 0.0001$). The male life span diminished at LC₂₅ level (ANOVA; $F(2, 48) = 4.94$, $P = 0.0114$) but the female life span at LC₁₀ and

LC₂₅ level was similar to the offspring (ANOVA; $F(2, 48) = 1.72$, $P = 0.1910$).

3.4. Oviposition Period in Parent and Offspring. Table 4 presents the adult longevity and preoviposition, oviposition, and postoviposition period of treated insects. In the parent generation, treatment with the LC₁₀ and LC₂₅ values of pyriproxyfen had no significant effect on adult preoviposition and postoviposition period as well as the male and female adult longevity (ANOVA; APOP, $F(2, 42) = 1.94$, $P = 0.1558$; postoviposition, $F(2, 42) = 0.92$, $P = 0.4057$; male adult longevity, $F(2, 42) = 1.87$, $P = 0.1662$; female adult longevity, $F(2, 42) = 0.40$, $P = 0.6759$). Also, there was a significant decrease in the oviposition period in the parent generation by sublethal doses (ANOVA; $F(2, 42) = 14.14$, $P < 0.0001$). Pyriproxyfen had no effect on the APOP (ANOVA; $F(2, 48) = 1.26$, $P = 0.2926$) and postoviposition period in the next generation (ANOVA; $F(2, 48) = 0.41$, $P = 0.6636$). The male longevity was significantly extended only by the LC₂₅ in offspring generation (ANOVA; $F(2, 48) = 2.48$, $P = 0.0421$). The female longevity at LC₂₅ level was lower than in the control in the parent generation (ANOVA; $F(2, 48) = 4.97$, $P = 0.0111$). Pyriproxyfen significantly enhanced the total preoviposition period in a dose-dependent manner (ANOVA; $F(2, 48) = 48.78$, $P < 0.0001$). Unlike the LC₁₀ dose, LC₂₅ decreased preoviposition period in the offspring generation (ANOVA; $F(2, 48) = 5.48$, $P = 0.0074$).

3.5. Sublethal Effects on Population Growth Parameters. Table 5 shows the effects of pyriproxyfen on population growth parameters of the DBM. Pyriproxyfen had no significant effect on the gross reproductive rate (GRR) (ANOVA; $F(2, 48) = 1.68$, $P = 0.1973$). The net reproductive rate (R_0) differed significantly between control and sublethal treatments (ANOVA; $F(2, 48) = 21.98$, $P < 0.0001$). Also, the intrinsic rate of increase (r_m) and finite rate of increase (λ) diminished by both concentrations (ANOVA; r_m , $F(2, 48) = 20.63$, $P < 0.0001$; λ , $F(2, 48) = 21.27$, $P < 0.0001$). Generation time (T) and doubling time (Dt) were significantly increased only at LC₁₀ compared to the control (ANOVA; generation time (T), $F(2, 48) = 8.90$, $P = 0.0005$; doubling time (Dt), $F(2, 48) = 4.47$, $P = 0.0166$). In addition, a significant difference was observed in birth rate (b) between control and LC₁₀ value. At LC₂₅, this was similar to control (ANOVA; $F(2, 48) = 7.94$, $P = 0.0010$). Death rate (d) was significantly decreased by LC₂₅ compared to the control; however, there was no observed significant difference in death rate between LC₁₀ and LC₂₅ (ANOVA; $F(2, 48) = 16.56$, $P < 0.0001$). Also Figures 2–4 show the life expectancy (e_x) (Figure 2), Age-specific survival rate (l_x) (Figure 3) and age-specific fecundity (m_x) (Figure 4) of DBM in control and treatment groups.

4. Discussion

The present proved that application of pyriproxyfen in leaf dip method has an effective way of suppressing the population of larval stages of the DBM. Topical effect of pyriproxyfen

TABLE 3: The effects of pyriproxyfen on the developmental period of 3rd instar larvae of *P. xylostella* in two subsequent generations.

Treatments	Developmental time (mean $\pm$ SE) (day) [#]			<i>F</i>	<i>P</i>	df _{t,e}
	Control	LC ₁₀	LC ₂₅			
Egg	2.18 $\pm$ 0.03 ^c	3.31 $\pm$ 0.04 ^b	3.54 $\pm$ 0.05 ^a	313.34	<0.0001	2, 281
Larva 1	2.98 $\pm$ 0.05 ^a	2.84 $\pm$ 0.14 ^{ab}	2.48 $\pm$ 0.14 ^b	7.01	0.0012	2, 151
Larva 2	1.61 $\pm$ 0.08 ^b	2.18 $\pm$ 0.19 ^a	2.00 $\pm$ 0.20 ^{ab}	3.83	0.0244	2, 122
Larva 3	1.25 $\pm$ 0.07 ^b	1.88 $\pm$ 0.24 ^a	1.85 $\pm$ 0.25 ^a	6.03	0.0032	2, 114
Larva 4	1.51 $\pm$ 0.06 ^c	3.25 $\pm$ 0.20 ^a	2.25 $\pm$ 0.37 ^b	37.65	<0.0001	2, 101
All larvae	7.44 $\pm$ 0.23 ^b	9.88 $\pm$ 0.28 ^a	8.31 $\pm$ 0.53 ^a	22.29	<0.0001	2, 99
Prepupa	0.40 $\pm$ 0.06 ^a	0.26 $\pm$ 0.08 ^a	0.25 $\pm$ 0.11 ^a	1.04	0.3559	2, 99
Pupa	3.70 $\pm$ 0.10 ^b	4.42 $\pm$ 0.17 ^a	4.46 $\pm$ 0.40 ^a	6.69	0.0019	2, 94
Pre adult stages	13.61 $\pm$ 0.19 ^c	18.11 $\pm$ 0.42 ^a	16.60 $\pm$ 0.77 ^b	52.37	<0.0001	2, 94
Total life span (male)	30.78 $\pm$ 1.26 ^a	31.78 $\pm$ 1.09 ^a	23.71 $\pm$ 1.92 ^b	4.94	0.0114	2, 48
Total life span (female)	31.30 $\pm$ 1.18 ^a	32.90 $\pm$ 2.15 ^a	27.44 $\pm$ 2.23 ^a	1.72	0.1910	2, 48

indicates that means marked with the same letters within a row are not significantly different (Tukey's test; $P < 0.05$).

TABLE 4: The effects of sublethal concentrations of pyriproxyfen on preoviposition, oviposition, and postoviposition periods and adult longevity of *P. xylostella* in parent and offspring generations.

Generations	Stages	Mean $\pm$ SE (day) ^A			<i>F</i>	<i>P</i>	df _{t,e}
		Control	LC ₁₀	LC ₂₅			
Parent	APOP ^B	2.13 $\pm$ 0.66 ^a	5.13 $\pm$ 1.11 ^a	7.73 $\pm$ 2.25 ^a	1.94	0.1558	2, 42
	Oviposition	11.26 $\pm$ 0.95 ^a	5.73 $\pm$ 1.13 ^b	4.06 $\pm$ 0.90 ^b	14.14	<0.0001	2, 42
	Postoviposition	0.86 $\pm$ 0.30 ^a	1.66 $\pm$ 0.39 ^a	1.73 $\pm$ 0.80 ^a	0.92	0.4057	2, 42
	Male adult longevity	20.07 $\pm$ 1.98 ^a	17.46 $\pm$ 1.14 ^a	15.73 $\pm$ 1.54 ^a	1.87	0.1662	2, 42
	Female adult longevity	14.26 $\pm$ 1.16 ^a	12.53 $\pm$ 1.05 ^a	13.53 $\pm$ 1.81 ^a	0.40	0.6759	2, 42
Offspring	APOP ^B	4.13 $\pm$ 0.78 ^a	6.45 $\pm$ 1.56 ^a	4.55 $\pm$ 1.67 ^a	1.26	0.2926	2, 48
	TPOP ^C	13.17 $\pm$ 0.26 ^b	22.63 $\pm$ 1.51 ^a	21.33 $\pm$ 2.04 ^a	48.78	<0.0001	2, 48
	Oviposition	10.65 $\pm$ 0.89 ^a	7.18 $\pm$ 2.00 ^{ab}	4.44 $\pm$ 1.49 ^b	5.48	0.0074	2, 48
	Postoviposition	2.06 $\pm$ 0.57 ^a	1.27 $\pm$ 0.35 ^a	1.33 $\pm$ 1.21 ^a	0.41	0.6636	2, 48
	Male adult longevity	16.60 $\pm$ 1.27 ^b	13.50 $\pm$ 1.18 ^b	20.71 $\pm$ 4.42 ^a	2.48	0.0421	2, 48
	Female adult longevity	16.62 $\pm$ 1.20 ^a	14.90 $\pm$ 1.87 ^a	10.33 $\pm$ 2.32 ^b	4.97	0.0111	2, 48

A: means marked with the same letters within a row are not significantly different (Tukey's test; $P < 0.05$).

B: adult preoviposition period, time between adult emergence and first oviposition.

C: total preoviposition period, time from birth to first reproduction in female.

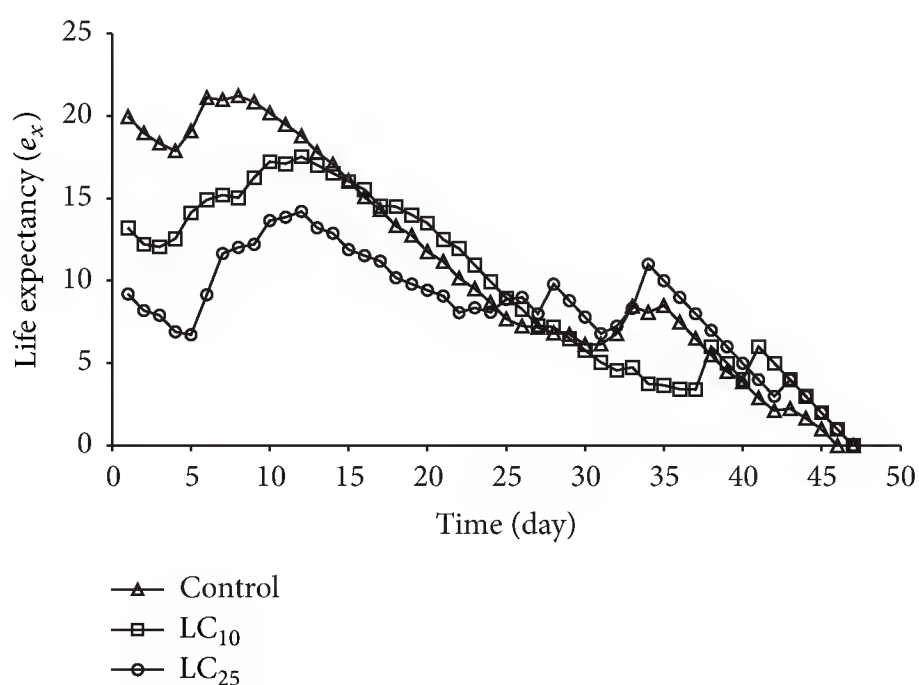
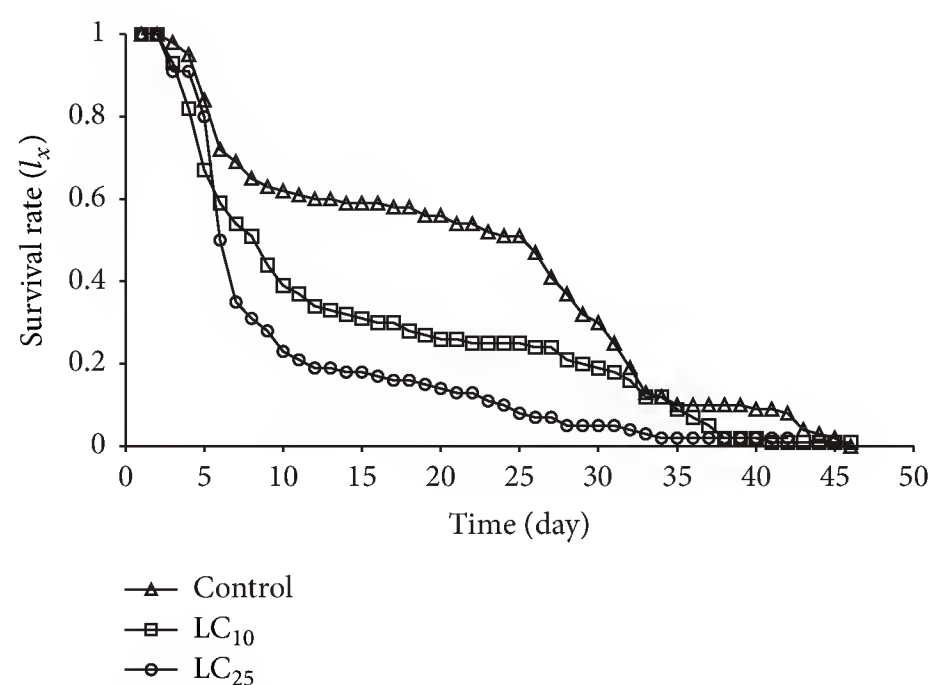
FIGURE 2: The life expectancy (e_x) of *P. xylostella* after treatment with sublethal doses of pyriproxyfen.FIGURE 3: The survival rate (l_x) of *P. xylostella* treated with sublethal doses of pyriproxyfen.

TABLE 5: The effects of pyriproxyfen on the biological parameters of 3rd instar larvae of *P. xylostella* in the next generation.

Treatment	Mean $\pm$ SE [#]							
	GRR	R_0	r_m (day ⁻¹)	λ (day ⁻¹)	T (day)	Dt (day)	b (birth rate)	d (death rate)
Control	98.68 $\pm$ 12.02 ^a	50.76 $\pm$ 4.00 ^a	0.190 $\pm$ 0.008 ^a	1.21 $\pm$ 0.010 ^a	20.73 $\pm$ 0.71 ^b	3.61 $\pm$ 0.18 ^b	0.26 $\pm$ 0.01 ^a	0.07 $\pm$ 0.003 ^b
LC ₁₀	76.14 $\pm$ 17.69 ^a	12.99 $\pm$ 1.85 ^b	0.099 $\pm$ 0.010 ^b	1.10 $\pm$ 0.011 ^b	26.44 $\pm$ 1.61 ^a	6.67 $\pm$ 1.04 ^a	0.17 $\pm$ 0.01 ^b	0.08 $\pm$ 0.003 ^b
LC ₂₅	45.64 $\pm$ 18.24 ^a	8.16 $\pm$ 2.02 ^b	0.112 $\pm$ 0.021 ^b	1.11 $\pm$ 0.022 ^b	21.08 $\pm$ 1.33 ^b	3.98 $\pm$ 0.08 ^{ab}	0.21 $\pm$ 0.02 ^{ab}	0.10 $\pm$ 0.003 ^a
F	1.68	21.98	20.63	21.27	8.90	4.47	7.94	16.56
P	0.1973	<0.0001	<0.0001	<0.0001	0.0005	0.0166	0.0010	<0.0001
df _{t,e}	2, 48	2, 48	2, 48	2, 48	2, 48	2, 48	2, 48	2, 48

#: means marked with the same letters within a column are not significantly different (Tukey's test; $P < 0.05$).

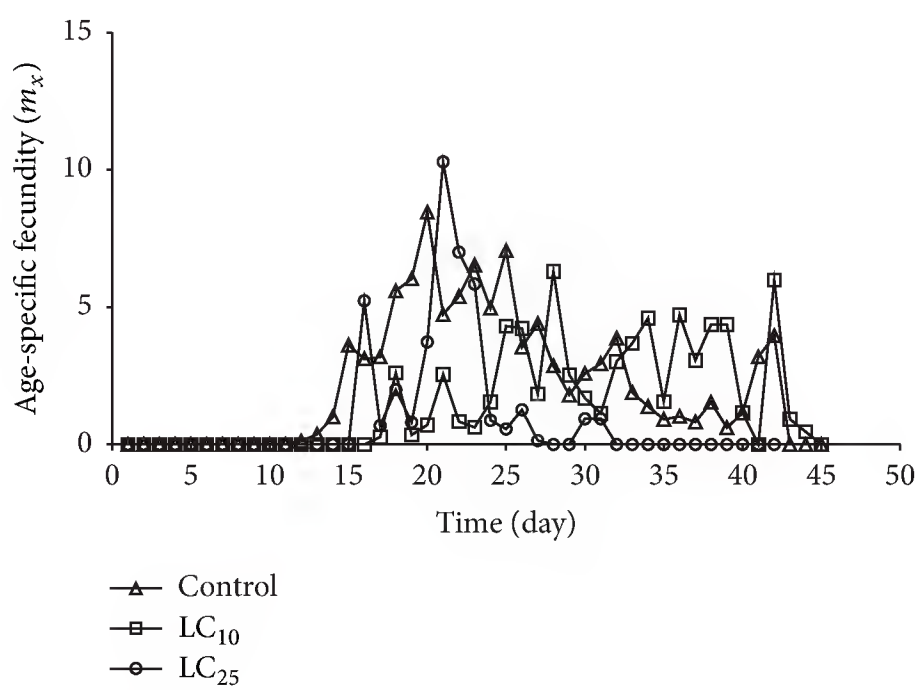


FIGURE 4: Age-specific fecundity (m_x) of *P. xylostella* treated with pyriproxyfen.

on DBM was evaluated in a previous study [26]. Toxicity of pyriproxyfen in leaf dip method in this study was 2-fold higher than in the topical method [26]. In leaf dip, the insecticide took effect orally and by contact, whereas its effect was by contact alone in topical method. This may be due to the increased toxicity in the leaf dip test.

The effects of pyriproxyfen were determined on fecundity in both generations in the current study. In previous studies, impact of pyriproxyfen on fecundity had various results [30, 31]. Steigenga et al. [30] reported an increase in fecundity when females of *Bicyclus anynana* (Butler) (Lepidoptera: Nymphalidae) were treated with sublethal doses of pyriproxyfen. On the contrary, pyriproxyfen at sublethal concentrations lowered the fecundity of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) [31]. The current results are consistent with those of Boina et al. [31] on *D. citri* which indicated a decrease in the fecundity by pyriproxyfen. In contrast, Abo-Elghar et al. [32] indicated a decrease in egg hatchability of *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) after treatment with sublethal doses of pyriproxyfen. However, in the current study, treatment with pyriproxyfen had no influence on egg hatchability of DBM. After treatment of the third instars with the LC₁₀ and LC₂₅ concentrations of pyriproxyfen, the surviving pupae showed a lower mean weight in treatment groups compared

to control in both (parent and offspring) generations. This weight reduction may be as result of decrease in nutrition ability after treatment with pyriproxyfen. Contrary to the current research, the body weight of tobacco budworm, *Heliothis virescens* F. (Lepidoptera: Noctuidae) increased by another JH mimic, fenoxycarb, compared to control [33]. The results are similar to those of Sial and Brunner [8] on *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) and cotton leaf worm *Spodoptera littoralis* (F.) (Lepidoptera: Noctuidae) treated with pyriproxyfen [33]. Contrary to our results, Mauchamp et al. [34] found an increase in the body weight of tobacco budworm, *Heliothis virescens* F. (Lepidoptera: Noctuidae) compared to control, after treatment with another JH mimic, fenoxycarb.

In addition, the current study showed an increase in the developmental time of eggs, larvae, and pupae of *P. xylostella* by sublethal concentrations of pyriproxyfen. A previous study reported that pyriproxyfen extended the larval developmental period of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) [33]. Similarly, Ghasemi et al. [35] also showed that the growth duration of *P. interpunctella* was postponed by pyriproxyfen. In contrast, pyriproxyfen at 50 and 150 mg L⁻¹ concentrations decreased the developmental times of 1st and 4th nymphal instars of soybean aphid, *Aphis glycines* Matsumura (Homoptera: Aphididae) [36].

From the results of the current study, sublethal doses did not change the adult longevity of parent generation. In the next generation, adult longevity increased in males and decreased in females. Application of pyriproxyfen on *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) decreased adult longevity [35]. Lee et al. [37] observed that treatment with pyriproxyfen significantly lowered the adult longevity of *Bemisia tabaci* (Homoptera: Aleyrodidae). Female longevity of *B. anynana* was similar to control after pyriproxyfen treatment [30]. Treatment with pyriproxyfen also affected other aspects of adult performance. In this study, the oviposition period of *P. xylostella* were shortened in both generations. Compared with the control, the total preoviposition period (TPOP) of offspring was increased upon application of pyriproxyfen treatment. According to the present results, pyriproxyfen delayed the maturation period of ovaries, resulting in postponement of mating by adults. Reduced oviposition period lead to a decrease in fecundity (number of eggs laid). Some of the biological parameters such as r_m , R_0 , and λ are related to fecundity. Therefore, suppression of this

ability is an important factor in IPM strategies. The current finding is similar to those of Zhang et al. [38] indicating that metaflumizone at LC₁₅ and LC₂₅ doses significantly increased TPOP of DBM. In contrast, other studies have provided mixed results. In some cases, the sublethal doses of insecticide have been found to either have no effect [37] or extend [26, 39] the preoviposition period in many insect groups. For example, Lee et al. [37] reported that treatment of *B. tabaci* with pyriproxyfen did not change the preoviposition period when compared to control [36]. An extension of the preoviposition period of DBM was observed after treating the larvae with cantharidin. In this study, the oviposition period of DBM declined in parent and *F1* generations. Also postoviposition period reduced in treated groups compared to control. In current study, oviposition in the females began after 7 days at LC₂₅, in parent generation. On the other hand, a decrease of 82% was observed in the fecundity by LC₂₅ when compared to the control. The observed preoviposition period and corresponding decrease in the fecundity in contact toxicity (LC₂₅) were 1.5 days and 56%, respectively [26].

Finally, pyriproxyfen at the sublethal concentrations had effect on biological parameters of DBM. Similar results have been found in previous studies [40, 41]. For example, Ahmad et al. [40] showed that Neemarin, at various concentrations, lowered finite rate of increase (λ), intrinsic rate of increase (r_m), and net reproductive rate (R_0) of DBM [40]. Also, Mahmoudvand and Moharramipour [41] observed that fenoxycarb increased Dt and declined R_0 , λ , and r_m of the DBM females. Han et al. [22] reported the Dt and T of DBM increased when treated with sublethal concentrations of chlorantraniliprole.

Contrary to the current study data, Zanuncio et al. [25] reported that R_0 , r_m , and λ of *Supputius cincticeps* (Stål) (Heteroptera: Pentatomidae) topically exposed to five doses of permethrin were raised compared to the untreated group.

In conclusion, from the results, it can be seen that pyriproxyfen, at sublethal doses, is effective in controlling the population of *P. xylostella*. However, the toxicity of JHAs was evaluated through topical method, and the current results showed that using the leaf dip can be a suitable method for this insecticide. Therefore sublethal doses of insecticides usage could be a good method for controlling insect pests that are resistant to insecticides after a few generations. Using high doses is the main cause of insect resistance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Behavioral Response of Plum Curculio, *Conotrachelus nenuphar*, to Synthetic Fruit Volatile Lures and the Male-Produced Aggregation Pheromone

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Plum curculio, *Conotrachelus nenuphar* Herbst (Coleoptera: Curculionidae), is a key pest of stone and pome fruits in North America. The behavioral response of adults of different physiological states (sex, age, diet, and mating status) to three commercially available synthetic lures, benzaldehyde (BZ) and plum essence (PE), the male-produced aggregation pheromone, grandisoic acid (GA), or their combinations, was studied in the laboratory. Four choice olfactometer bioassays demonstrated significant attraction of both sexes to PE lure. Both BZ and GA lures were not attractive to plum curculio when tested as commercially formulated. PE had higher release rate (1.51 mg/hr) than BZ (0.36 mg/hr) and GA (ca. 0.04 mg/hr), suggesting that the higher attractiveness of PE may be due to its relatively higher release rates. Tests with combined lures showed a neutral effect of combining GA with PE and an inhibitory effect of combining BZ with PE. The physiological conditions of the weevils had no significant effect on their response to the lures. Olfactometer tests with pure 1,2,4-trichlorobenzene (TCB) confirmed that this compound was inhibitory to plum curculio. These results are discussed in relation to the contrasting field reports which implicated BZ + GA as an effective attractant for plum curculio.

1. Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), is a major pest of many stone and pome fruit crops, which is widely distributed over the United States (U.S.) and Canada, east of the Rocky Mountains [1–4]. It is the most important direct pest of peaches (*Prunus persica* L.) in the southeastern U.S. [5–9]. Adult weevils typically overwinter in wooded lots adjacent to orchards or around fence rows from where they immigrate into peach orchards in the spring beginning around bloom [7, 10–12]. Like many insects, plum curculio uses olfactory cues, specifically host fruit derived volatiles, to locate host fruit [13–19]. Earlier studies identified several active compounds from host plants (both fruiting and nonfruiting parts) which are attractive to plum curculio [16, 17, 20, 21]. To date, the most attractive

plant-based volatiles for plum curculio are benzaldehyde (BZ) and foliar and woody tissue of plum trees [16, 21, 22]. Benzaldehyde has since been formulated as an attractant for plum curculio and is commercially available as a lure. Plum essence (PE), a commercially available synthetic mixture of plant essence, is also an attractant for plum curculio [23, 24]. The male-produced aggregation pheromone of plum curculio, grandisoic acid (GA), which was identified by [25], was later shown to act synergistically with BZ to increase adult trap captures in the field [26, 27]. Consequently, a combined lure consisting of BZ and GA was developed in Massachusetts, in USA, [26] and Quebec, in Canada, and remains to date the most widely used attractant for monitoring plum curculio in orchards [22, 26, 28–30]. The attractiveness of benzaldehyde + GA has, however, been shown to be consistently high in apple orchards in the Northeast but

less so in peach orchards in the mid-Atlantic region [29]. Also, captures of weevils in traps baited with this combined lure or other synthetic attractants can decline rapidly after fruit set due to intense competition from volatiles released by rapidly developing fruit [18, 29]. A recent study in Alabama peach orchards showed that captures of plum curculio in pyramid (also called Tedders trap) and “Circle” or screen traps (the two most popular traps for plum curculio) were improved numerically by the addition of BZ, PE, or GA alone (single lures) and were significantly enhanced only by the addition of the combined BZ + PE lure [9]. The popular combined BZ + GA lure was significantly less attractive than the combined BZ + PE lure. The results suggested an additive interaction between BZ and GA, in contrast to the synergistic interaction reported in Massachusetts apples [26, 30]. These varying results on lure performance may be related to several factors including the type of insect (strains), physicochemical properties of the lures, and differential attractiveness of tree fruit species/varieties, prevailing orchard conditions, strain differences, and physiological state of weevils. Two strains of plum curculio, namely, the northern and southern strains, are known to occur in continental North America. The northern strain has an obligate adult reproductive winter diapause, with one generation per year, whereas the southern strain weevils are multivoltine, with a facultative diapause. There is, therefore, the possibility that the olfactory response of adult plum curculios to synthetic lures may vary according to strain type (e.g., pheromone components produced by southern and northern strain plum curculios might differ). Also, the response to synthetic lures might differ depending on the habitat. For example, the response of plum curculio to synthetic lure has been studied mostly in peach orchards in the south and in apple orchards in the north (e.g., Massachusetts and Quebec). Also the physiological state of insects is known to play a major role in their response to odor cues [31–34]; however, we are not aware of any published studies on the response of plum curculio of different physiological states to currently available lures. This research was therefore designed to evaluate effects of physiological factors on the response of plum curculio to commercial lures. Specifically, we evaluated the response of weevils of different physiological status (sex, age, diet, and mating) to single and combined lures of BZ, PE, and GA in laboratory olfactometer bioassays. Based on the knowledge of the field ecology and spring and summer migration of plum curculio from overwintering sites, we tested the following key hypotheses: (i) females will show greater response to the lures than males, (ii) younger (preoviposition adults) weevils will show greater response to the lures than older (oviposition-postoviposition) weevils, (iii) starved weevils will show greater response to the fruit-based lures (BZ and PE) than fed weevils, and (iv) mating will have no significant effect on response of weevils to the lures. Next, the release rates of the lures were determined gravimetrically and used to provide explanations for the results recorded in the olfactometer bioassays. It is hoped that the results of this laboratory study will assist with the interpretation of field data on captures of plum curculio in traps baited with these lures.

2. Materials and Methods

2.1. Test Insects. The colony of plum curculio adults used for this study was maintained on pesticide-free green thinning apples in a growth chamber at $25 \pm 1^\circ\text{C}$, 65–70% RH, and 12:12 h (L:D) photoperiod. The weevils used to start the laboratory colony were collected from peach orchards in central Alabama and had been reared for more than 10 generations before the tests. The colony was periodically supplemented with weevils collected from the same field locations in Alabama. The rearing procedures followed that of [35, 36]. Females and males were separated using the methods of [37] and then tested separately.

2.2. Lure Treatments. The lures evaluated were commercial liquid formulations of benzaldehyde (BZ), plum essence (PE), and grandisoic acid (GA). The BZ and PE lures were obtained from Great Lakes IPM (Vestaburg, MI), while the GA lure was obtained from ChemTica International (San Jose, Costa Rica). The BZ and PE lures were in liquid form and were used without any major modifications through either dilution or mixing with solvents. The GA lure dispenser consisted of a heat-sealed polymer membrane.

2.3. Four-Choice Olfactometer Bioassays. A four-choice olfactometer (Analytical Research Systems, Gainesville, FL) was used to determine the behavioral response of adult plum curculio of different physiological states to the above lures. The four-choice olfactometer system was similar to that described by [38, 39]. Briefly, the apparatus consisted of a central chamber ($30 \times 30 \times 5$ cm) with orifices (arms) at the four corners through which purified and humidified air can be drawn in, creating four potential odor fields, and a central orifice where mixing of the airflow from the arms occurred. In the two experiments described below, only two (adjacent to each other) of the four orifices were designated for lure treatments while the other two adjacent orifices were designated for control (i.e., blank dispenser). In other words, only two lure treatments (binary tests) were compared at a given time. Although the device is designed to use push air, we did not use push air in this study because preliminary experiments indicated that plum curculio responded better to the lures under minimal airflow (near still air) conditions, as has been previously reported [20]. This may be related to the tendency of plum curculio to feign death upon the slightest disturbance. The BZ and PE lures were dispensed by transferring aliquots into 0.8 mL microcentrifuge vials (USA Scientific Inc., Ocala, FL) in which a cotton string (~2.5 cm long) was threaded through a hole drilled through the lid of the cap to aid in the release of the lures. The GA lure dispenser consisted of a heat-sealed polymer membrane to protect the pheromone. The membrane was removed before placing the dispenser in the olfactometer arm. The lure treatments were placed in their designated olfactometer arms for 20 min prior to release of test weevils to ensure the stabilization of the diffusion of the lures.

2.4. Olfactometer Response of Plum Curculio to Commercial Lures. Two separate experiments were conducted to evaluate

response of plum curculio to the three tested lures. In the first experiment, single lures were evaluated as binary or paired treatments (i.e., BZ versus PE, BZ versus GA, and PE versus GA). In the second experiment, the most attractive single lure determined in the first experiment (i.e., PE) was selected and compared against combined (two component) lure treatments in binary tests (i.e., PE versus BZ + PE, PE versus BZ + GA, and PE versus PE + GA) to determine the type of interaction (i.e., additive, synergistic, neutral, or negative/inhibitory) between any two lures. Weevils of different physiological states such as food deprivation (starved for 24 h versus fed ad libitum), age (preoviposition adults or 10–14-day olds versus oviposition-postoviposition adults or 20–24-day olds), and mating status (unmated versus mated) were tested in both experiments to determine the effect of the above physiological factors on the response of plum curculio to the lures. We considered 24 hr starvation to be enough to generate “hungry” weevils following [15, 16]. These resulted in a total of eight physiological treatments per sex. Groups of five females or males of each physiological treatment were released in the olfactometer and replicated six times per sex. Released weevils were given 30 min to respond by walking into one of the four olfactometer arms. Those that did not make a choice within this period were considered as “nonresponders” and excluded from the test. A weevil was used only once. At the end of the test with each physiological treatment, the olfactometer set-up was rinsed with soap, water, and acetone. The glassware was then heated in an oven to about 200°C for ~6 h before reuse. The position of each lure in the olfactometer was alternated after each replicate (i.e., lures were reassigned to different olfactometer arms) to minimize position effect. All bioassays were carried out under red light in a dark room at $25 \pm 1^\circ\text{C}$, $55 \pm 10\%$ RH between 1600 and 2400 h local time from June 2008 to December 2009. The time of day chosen was based on previous report which showed plum curculio adults to be most active in the field during scotophase [40, 41]. Data from each experiment were first analyzed by using standard least square analysis of variance (ANOVA), a model capable of determining the effects of multiple factors, to test for effects of sex (male versus female), age (preoviposition adults or 10–14-day olds versus oviposition-postoviposition adults or 20–24-days olds), diet (fed versus starved for 24 hr), mating status (mated versus unmated), and lure treatments on the response of plum curculio. The model also allowed testing for effects of single (one-way), double (two-way), triple (three-way), ternary (four-way), and five-way interactions (a total of 31 possible interactions) (Table 1) among the five factors. Because the model showed no significant effect of age, diet, mating status, or interactions among the majority of the factors on response of plum curculio to the lure treatments (Table 1), data for these physiological treatments were pooled for each sex and analyzed using one-way ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) test to determine significant differences in the response of each sex to the different lure treatments [42].

2.5. Olfactometer Response of Plum Curculio to Synthetic Chemical Components in Commercial Lures. Olfactometer

bioassays were conducted to evaluate the response of plum curculio to the key chemical components of the BZ and PE lures which are BZ and TCB. The aim was to determine the biologically active chemical components in each lure. The synthetic pure compounds identified in each lure were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and tested against the commercial lures in four-choice olfactometer bioassays using the procedures described above for the lures. One difference was that three odor treatments were compared simultaneously (i.e., multiple treatment comparisons). For this, three of the four arms of the olfactometer were designated for odor treatments, while the remaining arm was designated as hexane control. Two experiments were conducted each comparing three odor treatments versus control consisting of empty microcentrifuge vials. In experiment 1, pure benzaldehyde (BEN) and pure 1,2,4-trichlorobenzene (TCB), the two key chemical components identified in the commercial BZ lure, were compared against BZ lure. The dose of BEN and TCB tested in the olfactometer was similar to the amount of each compound detected in BZ lure by GC-MS. Based on the results of the above first experiment we hypothesized that TCB, which is commercially formulated with BZ lure as a stabilizing agent (but not formulated with PE lure), is likely the compound responsible for the relatively lower attractiveness of BZ lure compared to PE lure. To test this hypothesis regarding “repellent” or “dampening” effect of TCB when formulated with the BZ or PE lure, a second experiment was conducted which compared PE lure (found to be highly attractive in the previous lure tests), pure TCB, and mixture of PE and TCB (PE + TCB). If TCB truly has a repellent or inhibitory effect, we would expect the combined PE + TCB treatment to be less attractive than PE lure. TCB was tested singly with PE at a dose similar to that detected in the BZ lure. In the mixed PE + TCB treatment, both compounds were released from separate vials placed in the assigned olfactometer arm. For each experiment, groups of five female or male weevils (>20 days old, mated, and starved for 24 h) were released in the olfactometer. The experiment was replicated 12 times per sex. Data for each sex were analyzed using one-way ANOVA followed by the Tukey-Kramer HSD test to determine significant differences in the response of female or male weevils to the different odor treatments [42].

2.6. Estimation of Release Rates of Commercial Lures and Synthetic Components. The release rates of the commercial lures (BZ and PE) and pure synthetic components identified as released by the lures (BEN/TCB for BZ lure and BEN for PE lure) were determined gravimetrically in the laboratory using the methods described by [29]. Briefly, ~0.8 mL of each treatment (i.e., BZ lure, PE lure, BEN, or TCB) was transferred into a microcentrifuge vial, which was then weighed on a microbalance (Ohaus Adventurer Analytical Balance; model AR2140, Central Carolina Scale, Sanford, NC). Each vial was then placed in one of the chambers of the four-choice olfactometer. The experiment was conducted at $25 \pm 1^\circ\text{C}$, $55 \pm 10\%$ RH between 1600 and 2400 h local time. The weight of each vial was determined again 24 h later. The experiment was repeated seven times using fresh vials and compounds

TABLE 1: Standard least squares analyses of variance testing for effects of age, diet, lure type, mating status, and sex and their interactions on response of *C. nenuphar* to single components of benzaldehyde (BZ), plum essence (PE), and grandisoic acid (GA).

Type of effect	Source of variation	d.f.	BZ versus PE			GA versus PE			GA versus BZ		
			MS	<i>F</i>	<i>P</i>	MS	<i>F</i>	<i>P</i>	MS	<i>F</i>	<i>P</i>
Single	Age	1	0.21	0.35	0.5524	0.09	0.13	0.7158	0.00	0.00	1.0000
	Diet	1	0.07	0.11	0.7413	1.26	1.78	0.1825	0.17	0.22	0.6360
	Lure type	3	465.03	259.93	<0.0001	367.65	173.52	<0.0001	52.30	23.47	<0.0001
	Mating status	1	0.02	0.04	0.8430	0.17	0.24	0.6275	0.09	0.13	0.7226
	Sex	1	0.07	0.11	0.7413	0.01	0.01	0.9034	0.17	0.22	0.6360
Double	Age * lure type	3	3.57	2.00	0.1145	3.43	1.62	0.1852	2.60	1.17	0.3217
	Diet * age	1	0.07	0.11	0.7413	0.04	0.06	0.8082	0.51	0.69	0.4077
	Diet * age * lure type	3	2.22	1.24	0.2957	1.81	0.86	0.4645	1.76	0.79	0.5001
	Diet * lure type	3	8.51	4.76	0.0029	2.43	1.15	0.3308	5.98	2.68	0.0468
	Mating status * age	1	0.59	0.98	0.3223	0.01	0.01	0.9034	0.00	0.00	1.0000
	Mating status * diet	1	0.07	0.11	0.7413	0.09	0.13	0.7158	0.67	0.90	0.3441
	Mating status * lure type	3	0.38	0.21	0.8867	2.85	1.35	0.2591	0.72	0.32	0.8090
	Sex * age	1	0.07	0.11	0.7413	0.04	0.06	0.8082	0.01	0.01	0.9058
	Sex * diet	1	0.07	0.11	0.7413	0.04	0.06	0.8082	0.09	0.13	0.7226
	Sex * lure type	3	7.05	3.94	0.0088	5.43	2.56	0.0549	7.35	3.30	0.0207
	Sex * mating status	1	0.21	0.35	0.5524	0.09	0.13	0.7158	0.67	0.90	0.3441
	Mating status * age * lure type	3	2.20	1.23	0.2999	1.68	0.79	0.4993	9.44	4.24	0.0059
Triple	Mating status * diet * age	1	0.1276	0.21	0.6440	0.04	0.06	0.8082	0.01	0.01	0.9058
	Mating status * diet * lure type	3	0.76	0.42	0.7362	7.84	3.70	0.0121	2.40	1.08	0.3597
	Sex * age * lure type	3	2.59	1.45	0.2287	5.98	2.82	0.0390	1.22	0.55	0.6505
	Sex * diet * age	1	0.07	0.11	0.7413	0.09	0.13	0.7158	0.00	0.00	1.0000
	Sex * diet * lure type	3	2.38	1.33	0.2639	3.48	1.64	0.1795	6.09	2.73	0.0437
	Sex * mating status * age	1	0.32	0.53	0.4678	0.04	0.06	0.8082	0.01	0.01	0.9058
	Sex * mating status * diet	1	0.07	0.11	0.7413	0.00	0.00	1.0000	0.51	0.69	0.4077
	Sex * mating status * lure type	3	2.07	1.16	0.3262	3.01	1.42	0.2366	3.19	1.43	0.2338
Ternary	Mating status * diet * age * lure type	3	1.07031	0.60	0.6166	2.56	1.21	0.3063	6.59	2.96	0.0325
	Sex * diet * age * lure type	3	2.09	1.17	0.3217	3.18	1.50	0.2146	0.15	0.07	0.9781
	Sex * mating status * age * lure type	3	0.51	0.28	0.8371	0.98	0.46	0.7089	0.14	0.06	0.9804
	Sex * mating status * diet * age	1	0.00	0.00	0.9474	0.01	0.01	0.9034	0.17	0.22	0.6360
	Sex * mating status * diet * lure type	3	4.47	2.50	0.0598	2.44	1.15	0.3289	2.18	0.98	0.4037
Five-way	Sex * mating status * diet * age * lure type	3	0.90	0.51	0.6790	2.18	1.03	0.3806	3.06	1.37	0.2505

Numbers in bold indicate effects which showed significant differences (ANOVA, Tukey-Kramer HSD, $P < 0.05$).

each time. The release rate of GA lure was also determined in a parallel study using the same procedures. The mean release rates (mg/day) were calculated for each lure and the synthetic form of their components. A one way ANOVA was performed followed by the Tukey-Kramer HSD test to determine significant differences in the release rates of the different lures and synthetic compounds [42].

3. Results

3.1. Olfactometer Response of Plum Curculio to Commercial Lures. Standard least squares analyses of the data from the first experiment (BZ versus PE binary test) revealed no significant effect of sex ($F = 0.11$, d.f. = 1, and $P = 0.7413$), age ($F = 0.35$, d.f. = 1, and $P = 0.5524$), diet ($F = 0.11$, d.f. = 1, and $P = 0.7413$), or mating status ($F = 0.04$,

d.f. = 1, and $P = 0.8430$) on plum curculio response (Table 1). However, the effect of lure was highly significant ($F = 259.93$, d.f. = 3, and $P < 0.0001$) (Table 1). The only two significant interactions recorded were sex $\times$ lure ($F = 3.94$, d.f. = 3, and $P = 0.0088$) and diet $\times$ lure ($F = 4.76$, d.f. = 3, and $P < 0.0029$) (Table 1). Similar results were obtained for the other binary treatment comparisons of single lures (i.e., BZ versus GA and PE versus GA) or mixed lures (i.e., PE versus BZ + PE, PE versus BZ + GA, and PE versus PE + GA). Based on these results which indicated that the physiological conditions of adult plum curculio have very little effect on their response to the lures, data obtained for weevils of different physiological states (i.e., age, diet, and mating) were pooled by sex and analyzed using one-way ANOVA to compare response of each sex to different lure treatments.

Analysis of the pooled data showed that when BZ and PE were compared as binary odor treatments, plum curculio

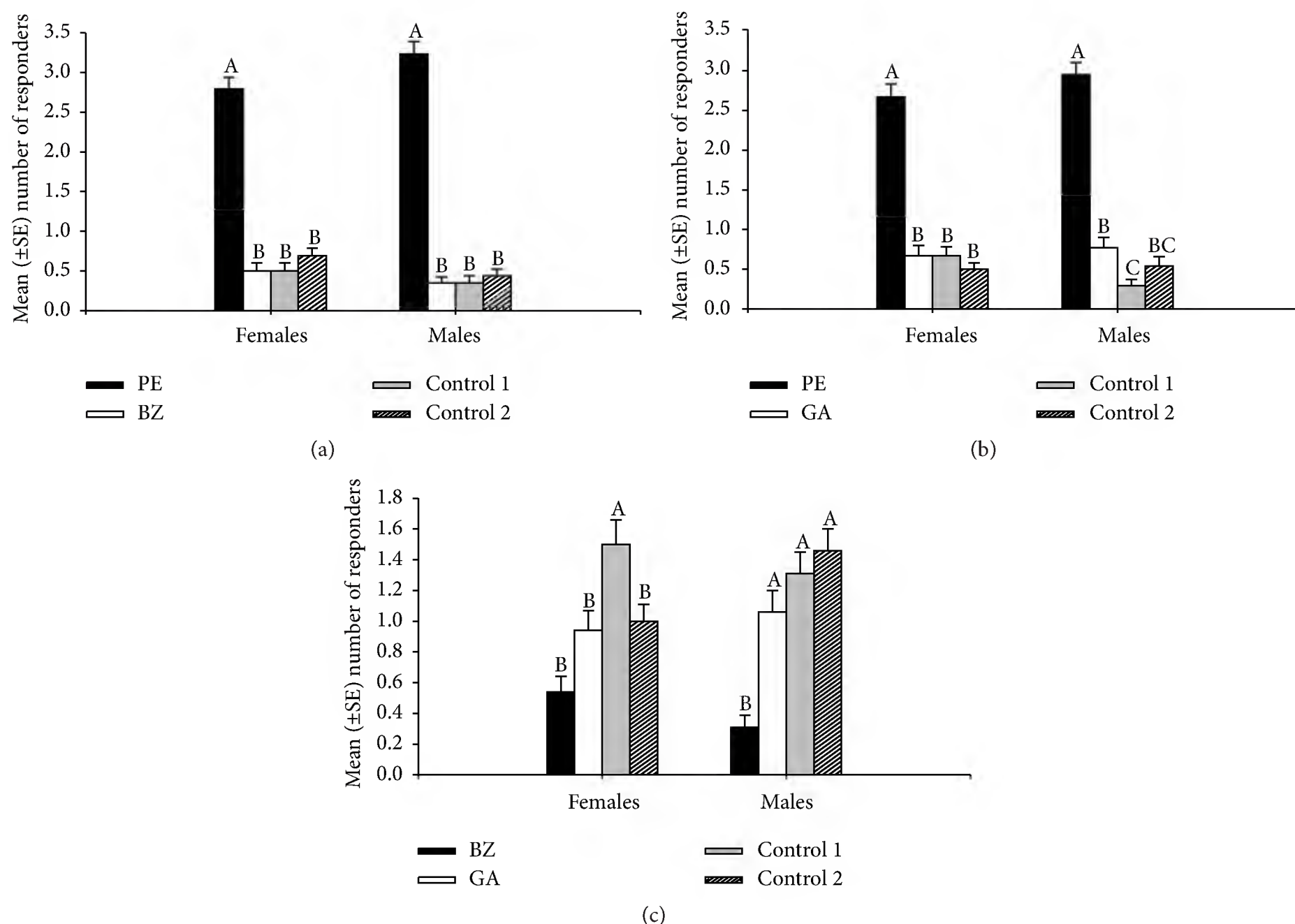


FIGURE 1: Response of plum curculio to commercial lures of benzaldehyde (BZ), plum essence (PE), and grandisoic acid (GA) in four-choice olfactometer bioassays. In each test, two lure treatments (binary test) and two controls were compared: (a) BZ versus PE; (b) PE versus GA; and (c) BZ versus GA. In this figure and Figure 2, Control 1 = air and Control 2 = empty microcentrifuge vial. Groups of five weevils of either sex were released per test in the olfactometer and replicated six times. Means for each sex having no letter in common are significantly different (ANOVA, Tukey-Kramer HSD, $P < 0.05$).

females ($F = 92.29$, d.f. = 3, and $P < 0.0001$) and males ($F = 175.6$, d.f. = 3, and $P < 0.0001$) were significantly more attracted to PE than to BZ or the controls (Figure 1(a)). Similarly, the results of the binary comparison of GA versus PE showed significantly greater attraction of females ($F = 69.06$, d.f. = 3, and $P < 0.0001$) and males ($F = 104.4$, d.f. = 3, and $P < 0.0001$) to PE than to GA or the controls (Figure 1(b)). The results of the binary comparison of BZ versus GA showed greater response of females to one of the controls than to BZ or GA ($F = 9.8$, d.f. = 3, and $P < 0.0001$), and no differences were recorded between BZ and GA (Figure 1(c)). Similarly, significantly fewer males responded to BZ compared to GA or the controls ($F = 16.4$, d.f. = 3, and $P < 0.0001$) (Figure 1(c)). Together, these results indicate the nonattractiveness of BZ and GA lures in our olfactometer bioassays.

The results of the second binary experiments in which PE was compared against a combined lure treatment (i.e., BZ + PE, BZ + GA, or PE + GA) confirmed the superior attractiveness of the PE lure (Figure 2). In the binary comparison of PE versus BZ + PE, both the females ($F = 89.5$, d.f. = 3, and $P < 0.0001$) and males ($F = 42.7$, d.f. = 3,

and $P < 0.0001$) showed greater response to PE than to BZ + PE or the controls (Figure 2(a)). Similarly, females ($F = 65.9$, d.f. = 3, and $P < 0.0001$) and males ($F = 76.4$, d.f. = 3, and $P < 0.0001$) were more attracted to PE than to BZ + GA or the controls (Figure 2(b)). Females ($F = 34.27$, d.f. = 3, and $P < 0.0001$) and males ($F = 45.56$, d.f. = 3, and $P < 0.0001$) also showed greater attraction to PE than to PE + GA or the controls (Figure 2(c)). Similar results were also obtained when PE was compared against a treatment consisting of the three lures (i.e., BZ + PE + GA; data not presented). In addition to confirming the superior attractiveness of PE, these results also showed the nonattractiveness of BZ not only as a single lure but also when combined with PE.

3.2. Olfactometer Response of Plum Curculio to Chemical Components in Commercial Lures. Four-choice olfactometer tests were conducted to evaluate response of plum curculio to multiple treatments comprising the lures and their key chemical components (i.e., 3 odor treatments and 1 control). In experiment 1, the following four treatments were compared: BZ lure, pure synthetic BEN, pure synthetic TCB, and control. The results showed that females ($F = 9.64$, d.f. = 3, and

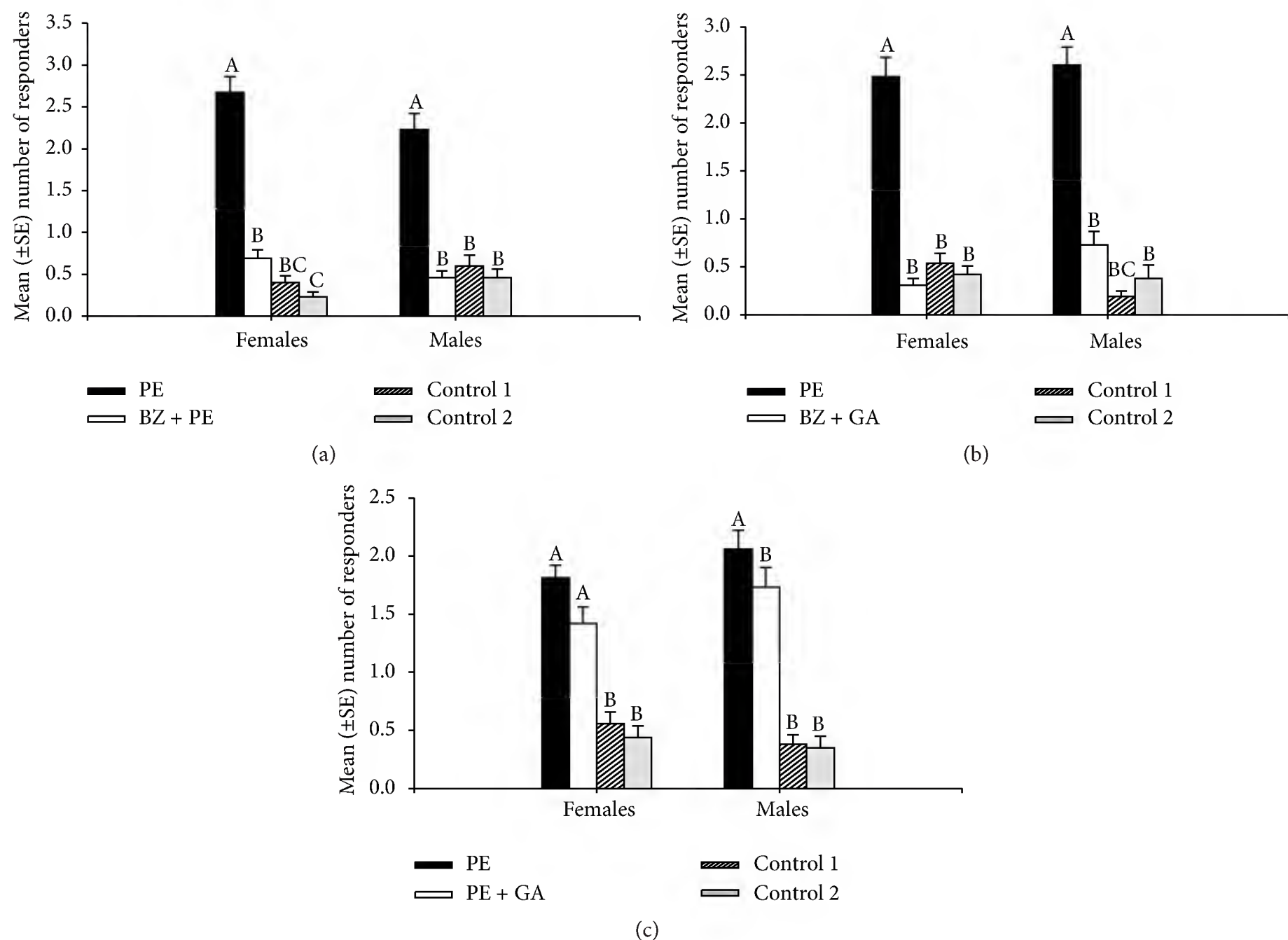


FIGURE 2: Response of plum curculio to combinations of commercial lures of benzaldehyde (BZ), plum essence (PE), and grandisoic acid (GA) in four-choice olfactometer bioassays. In each binary test, PE was compared against any two combinations of the three lures: (a) PE versus BZ + PE, (b) PE versus BZ + GA, and (c) PE versus PE + GA. Groups of five weevils of either sex were released per test in the olfactometer and replicated six times. Means for each sex having no letter in common are significantly different (ANOVA, Tukey-Kramer HSD, $P < 0.05$).

$P < 0.0001$) and males ($F = 6.87$, d.f. = 3, and $P < 0.0007$) were significantly more responsive to the control than to BZ lure or TCB (Table 2). Both sexes also showed slightly lower response to BEN than to the control. The results of experiment 2 in which PE was compared against TCB and PE + TCB showed that females were significantly more attracted to PE than to TCB or the control ($F = 17.27$, d.f. = 3, and $P < 0.0001$). Also, females were numerically more attracted to PE than to PE + TCB (Table 3). Similarly, males were significantly more attracted to PE than to the remaining treatments ($F = 12.50$, d.f. = 3, and $P < 0.0001$) (Table 2). These results again confirmed the attractiveness of the PE lure and the inhibitory effect of TCB when mixed with PE.

3.3. Estimation of Release Rates of Commercial Lures and Synthetic Components. Significant differences in release rates were recorded among the lures and compounds (i.e., BEN, BZ, PE, and TCB) ($F = 60.66$, d.f. = 3, and $P < 0.0001$). The gravimetric release rate of PE was much higher than the release rates of the other treatments (Table 3).

4. Discussion

The results of this laboratory study confirmed the attractiveness of the commercial plum essence lure (PE) (a synthetic mixture of plant essence) to plum curculio. Of the tested lures and synthetic components, PE was by far the most attractive to both sexes. This finding is in agreement with previous reports of PE as an attractant for plum curculio in the field [9, 23, 24]. The relatively greater attractiveness of PE lure may be due to its higher release rates, as determined in the release rate experiment. Our results, however, showed that the commercial benzaldehyde (BZ) lure was not attractive to plum curculio in olfactometer bioassays. This somewhat surprising finding is in contrast to the results of field studies which demonstrated attraction of plum curculio to traps baited with benzaldehyde [17]. In fact, BZ lure is commonly regarded as the most attractive lure for plum curculio when combined with GA and is widely used for monitoring the pest in the field [17, 26, 28, 43]. The third commercial lure tested, grandisoic acid (GA), which is the male-produced aggregation pheromone of plum curculio [25] was also not

TABLE 2: Response of *C. nenuphar* to the tested commercial lures and synthetic pure compounds in four-choice olfactometer bioassays.

Experiment	Compounds/lure treatments	Mean ($\pm$ SE) number of responders	
		Female	Male
1	BZ	0.25 $\pm$ 0.10 c	0.50 $\pm$ 0.15 b
	BEN	1.17 $\pm$ 0.24 ab	0.92 $\pm$ 0.19 ab
	TCB	0.58 $\pm$ 0.15 bc	0.58 $\pm$ 0.15 b
	Control	1.83 $\pm$ 0.32 a	1.50 $\pm$ 0.19 a
		$F_{3,44} = 9.64$	$F_{3,44} = 6.87$
		$P < 0.0001$	$P < 0.0007$
2	PE	2.08 $\pm$ 0.26 a	2.42 $\pm$ 0.38 a
	TCB	0.17 $\pm$ 0.11 b	0.33 $\pm$ 0.19 b
	PE + TCB	1.42 $\pm$ 0.19 a	0.92 $\pm$ 0.19 a
	Control	0.58 $\pm$ 0.19 b	0.92 $\pm$ 0.19 b
		$F_{3,44} = 17.27$	$F_{3,44} = 12.50$
		$P < 0.0001$	$P < 0.0001$

BZ: commercial benzaldehyde lure; BEN: pure synthetic benzaldehyde; TCB: pure synthetic 1,2,4-trichlorobenzene; PE: commercial plum essence lure. For each experiment, groups of 5 weevils of either sex were released per test in the olfactometer and replicated 12 times. For each experiment and sex, means having no letter in common are significantly different (ANOVA, Tukey-Kramer HSD, $P < 0.05$).

TABLE 3: Release rates of the tested commercial lures and synthetic pure compounds under laboratory conditions.

	Mean ($\pm$ SE) release rates mg per hour
BZ	0.36 $\pm$ 0.05 b
PE	1.51 $\pm$ 0.13 a
BEN	0.29 $\pm$ 0.09 b
TCB	0.08 $\pm$ 0.01 b
GA*	ca. 0.041

BZ: commercial benzaldehyde lure; PE: commercial plum essence lure; BEN: pure synthetic benzaldehyde; TCB: pure synthetic 1,2,4-trichlorobenzene; GA: grandisoic acid. Means having no letter in common are significantly different (ANOVA, Tukey-Kramer HSD, $P < 0.05$; $n = 5$). *Release rate was calculated based on manufacturer's recommendation.

attractive to both sexes when tested either as single lure or in combination with other lures. The results of the experiments in which combined lures were tested further confirmed the superior attractiveness of PE lure, which was more attractive than any combinations of the three lures. Combining BZ or GA lure with PE lure resulted in reduced attractiveness of PE lure. The data actually suggest a repellent or inhibitory effect of BZ when combined with PE and a neutral effect of combining GA with PE. These results are again contrary to some field studies which reported either a synergistic effect of combining BZ or GA lures [26] or an additive interaction between BZ and PE and between BZ and GA [9]. The combined BZ + GA lure is presently the most widely used attractant for monitoring plum curculio in orchards [22, 26, 28–30]. Also, a recent field study by our group showed that the combined BZ + PE was the most effective lure for monitoring plum curculio in Alabama peaches [9]. However, both combined lures (BZ + GA and BZ + PE lures) were not as attractive as the single PE lure in the present study. The difference between the results of this laboratory study and the above field reports may be related to differences between experimental conditions and other factors

such as the release rates of the lures. Chemical compounds such as the lures tested in this study, which depend on prevailing environmental conditions to be released in the right concentration, are likely to vary in their performance in fluctuating field conditions compared to the more stable laboratory environment. Intriguingly, the results of the GC-MS analyses of the lures showed that benzaldehyde (BEN) was the major component released from both BZ and PE lures (Akotsen-Mensah et al., unpublished). Why then is PE lure more attractive than BZ lure? The answer to this question is possibly related to the presence of 1,2,4-trichlorobenzene (TCB) in BZ lure. TCB is formulated with BZ lure as a stabilizing agent and was the only additional component released from BZ lure. TCB, however, was not detected in PE lure. This led us to hypothesize that the reduced attractiveness (or inhibitory effect) of BZ lure was due to TCB. We tested this hypothesis by comparing attraction of plum curculio to PE, TCB, and PE + TCB. The results indicated reduced attraction of plum curculio to PE + TCB compared to PE alone. However, it was unclear if TCB actually played a role in the observed nonattractiveness of BZ lure since the weevils were also not attracted to pure synthetic BEN. There is currently no evidence which suggests that TCB is a component of the general volatiles complex released by any of the host plants of plum curculio. Hence, we can only speculate that plum curculio is not likely to have evolved the ability to respond to TCB. However, the data which showed a threefold reduction in the response of both sexes to TCB compared to the control may suggest the possibility of an inhibitory effect of TCB. The reduced attractiveness of the combined PE + TCB treatment relative to PE further supports this possibility. Although BEN has been shown to degrade rapidly to benzoic acid and trans-stilbene in the laboratory [22], we did not detect these compounds in our analyses when BZ was exposed in the laboratory for up to 48 hours. Therefore, the nonattractiveness of BZ lure and BEN could not be attributed to degradation to benzoic acid

and other compounds. Our preliminary result using GC-MS analyses which showed that BEN was detected in greater amounts in BZ lure than in PE lure are not as relevant as the release rate results which showed that PE lure was released at a higher rate (~4-fold) than BZ lure (Akotsen-Mensah, unpublished). In general, the data showed no significant effect of physiological factors (i.e., age, diet, and mating) or sexual differences on the response of plum curculio to the tested lures. These results led us to reject most of our hypotheses and were somewhat surprising, in particular the null effect of diet on response. We had expected that plum curculio, which uses the same resources (fruit) for food and oviposition, will show greater response to fruit-based odor when starved than when fed. The results are in contrast to those reported by [15], which showed that starved weevils responded more than fed weevils to hexane extract of wild plum. However, it should be noted that the study by [15] used overwintering adults whose physiological conditions were largely unknown (with the exception of diet), whereas the weevils used in the present study were from a laboratory source and with known physiological conditions. Previous studies on the effect of physiological factors on response of other weevil species to odor have produced different results. While some studies have reported significant effect of some physiological factors on beetle response to host odor (e.g., [31–34, 44]), others have reported no effect [45]. In conclusion, the results of this laboratory study demonstrated significant attraction of plum curculio to PE lure. Contrary to field reports, GA lure was not attractive, while BZ lure was inhibitory. The difference between our results and previous field reports may be due to several factors. The inhibitory effects of BZ lure may be due to the presence of TCB. Aside from this, it is plausible that the weevils reared in the laboratory are different from those that occur naturally in the field in their response to odor. In addition, the northern strain of plum curculio tested in most of the field studies and the southern multivoltine strain tested in the present study may differ in their behavioral response to odor. Future studies are necessary to further determine the basis for the inhibitory effect of BZ lure recorded in this study.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Morphology of the Preimaginal Stages of *Lasioptera donacis* Coutin (Diptera: Cecidomyiidae), a Candidate Biocontrol Agent for Giant Arundo Cane

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The larval stages of *Lasioptera donacis* Coutin consist of three instars which develop within the mesophyll of the leaf sheaths of *Arundo donax* (L.) (Poaceae). The larvae feed aggregatively on mycelia of an ambrosia fungus. The third instars are similar to other members of the genus except for a three-pronged spatula (typically two-pronged) and five lateral papillae (typically four) and with a nonbristled first instar. A related species, *L. arundinis* (Schiner) which breeds on fungus in *Phragmites* (Poaceae), also has a three-pronged spatula and five lateral papillae but has a bristled first instar. The third instar of *L. donacis* has a feeding and a nonfeeding prepupal stage. Papillae associated with the spatula are sensory organs, sensilla chaetica, sensilla trichodea, and sensilla ampullacea, perhaps related to extraoral digestion of the fungal mycelia. Pupation occurs in the host plant within a silken cocoon. Egression of the adult is through an escape hatch excavated by the third instar.

1. Introduction

The genus *Lasioptera* Meigen 1818 is defined taxonomically by structures on the postabdomen and ovipositor of the adult female [1]. These structures are mycangia, organs which function in the transport of fungal conidia. The gall midges of this genus are obligate symbionts of a fungus on which the larval stages feed [2, 3]. Females collect the conidia and oviposit them with the eggs into the host plant. The fungus is an opportunistic endophyte capable of exploiting living plant hosts. The fungus rewards its symbiont for providing transport to potential host plants by providing nourishment in the form of an ambrosia, a filamentous mycelial mass which fills the tunnels inhabited by the larvae [4]. In these cases of symbiosis the inoculation of the fungus into the plant by the insect has been documented for the Platypodidae (Coleoptera) [5], the Siricidae (Hymenoptera) [6], and the Cecidomyiidae (Diptera) [3, 7]. Coutin and Faivre-Amiot [8] first discovered and outlined the tritrophic relationships among this insect, *Lasioptera donacis* Coutin 2001, the fungus, and its host plant *Arundo donax* L. (Poaceae: Poales). These authors also described and illustrated the ovipositor

with its mycangia on the adult female. The preimaginal stages have morphological features which adapt them to this resource niche and the objective of this study is to describe some of those adaptations.

General treatises on the morphology of larval cecidomyiids have been published by Otter [9], Möhn [10], Gagné [11–13], and Mamaev and Krivosheina [14]. The larval stages of the two most economically important cecidomyiids, the Hessian fly, *Mayetiola destructor* (Say 1817), and the sorghum midge, *Contarinia sorghicola* (Coquillett 1899), have been described in considerable detail primarily in the context of the damage to the host plant [15, 16]. Illustrations and descriptions of larval Lasiopteridi include Gagné et al. [17, 18] and Boe and Gagné [19]. The larvae of 20 species of *Lasioptera* were described in a taxonomic study by Möhn [10] which included the descriptions of new species based on larval specimens.

With few exceptions the larvae of *Lasioptera* spp. are narrowly host plant specific. Because *Arundo donax* (L.), the giant reed or carrizo cane, is an invasive plant that has spread into the riparian systems of the southern United States [20–22], *L. donacis* is under consideration as a potential biological control agent [23]. In that context, the basic biology

of this insect is of some importance and the morphological adaptations of the immature stages indicate the specific means by which it exploits the fungus within its host plant. A related fungus feeding species, *Lasioptera arundinis* (Schiner 1854), breeds in a related host, the common reed, *Phragmites australis* (Cav.) Trin. (Poaceae), and hence has a life cycle and morphology similar to that of *L. donacis*. Thus, a secondary objective of our study was to ascertain the differences in larval morphology between *L. donacis* as they appear in the original description by Coutin [24] and observed by us, with those of *L. arundinis* as described by Möhn [10] and Rohfritsch [4].

2. Materials and Methods

2.1. Rearing of *Lasioptera donacis*. The late instars and pupae examined in this study were dissected from host plant material collected near Montpellier, France (43°40'N; 04°01'E), and Vandellos, Spain (41°01'N; 0°50'E). These were shipped by air courier to the USDA-APHIS Arthropod quarantine facility near Edinburg, TX, where a research colony is maintained. Eggs and first instar specimens were collected by inducing oviposition by colony adults placed in cages with potted *Arundo* plants in a greenhouse with temperatures maintained at $23^{\circ} \pm 3^{\circ}\text{C}$. Similarly, RH was targeted to 100% but actual recorded RH varied around a mean of 80%. Light cycle was natural. According to Coutin and Faivre-Amiot [8] *Lasioptera* females seek preexisting holes in the leaf-sheath, made by a chloropid, for oviposition. Thus, in order to rear *L. donacis* we artificially perforated the leaf sheathes of potted plants with a fine pair of forceps. In our laboratory colonies all egg masses found were in the mesophyll channels underlying these artificial perforations. The colony at Edinburg was first established in 2007 and continues to the present day.

2.2. Preparation of Specimens. For chaetotaxy some larval specimens were cleared and mounted following the KOH-Clove oil method of Gagné [12] while others were cleared with d-limonene (Histoclear, National Diagnostics, Atlanta GA). Some of the cleared specimens were stained with aceto-orcein or eosin to enhance visualization. Measurements of larvae and larval structures were made on a Leica MZI6 dissecting microscope with Leica application suite software. Photomicrographs of third instars were made on a Nikon Eclipse E400 microscope. For first and second instars whole specimens were mounted on slides using Permount (Fisher Scientific, Hampton NH) and photographed with a Nikon SMZ645 microscope. For internal organs larvae were mounted in paraffin, sectioned with a rotary microtome, and stained with methylene blue or eosin. Chaetotaxy follows Gagné [12] and Möhn [10]. Nomenclature for the sensory organs is that of Zacharuk and Shields [25].

3. Results

Lasioptera donacis has three larval instars that are passed entirely within the spongy mesophyll layer of the stem leaf sheaths of *Arundo donax*. The larvae are essentially colonial, feeding in clusters of about the same stage of development,



FIGURE 1: First instars with a mass of black yeasty mycelium (center) at approximately ten days after oviposition. White hyphae are extending out from the old egg clutch into the plant tissues.

and presumably from the same clutch of eggs, but at times in larger aggregations that can include two instars likely from successive ovipositions. Hodin and Riddiford [26] demonstrated that the nutritional content of the mycelium was a factor in the inducement of paedogenesis in fungus feeding midge larvae. Characterized by a fourth instar that lacks the spatula, paedogenesis has not been reported in *Lasioptera*, nor did we observe this phenomenon in *L. donacis*.

Egg masses with fungal conidia were found in the medullary channels soon after exposure of the perforated plants to female midges. The first instars are free-living for at least several days after hatch because it typically requires about a week after inoculation for the dark mass of fungal mycelia to grow and accumulate (Figure 1). Invariably the second and early third instar larvae were found in channels of the medullary tissue, typically with the head end immersed in, and presumably feeding on, the dark yeasty mass of mycelia (Figure 2). The third instar has a feeding and a prepupal nonfeeding stage. At the end of the feeding stage the third instar spins a silken cocoon in which it will eventually pupate, still occupying the fungus filled channels of the leaf sheath mesophyll. The nonfeeding stage is found in the cocoon which is open at one end. Just prior to spinning the cocoon the larva cuts an escape hatch in the leaf wall from which the imago will eventually egress. It uses a sclerotized armature on the prothorax, common to almost all cecidomyiids, the “sternal spatula,” to excavate the emergence hole [26]. However, this hole does not open freely to the exterior; rather, a thin flap of epidermal plant tissue seals the hole. The larva then closes the cocoon by spinning a disc shaped, silken operculum. When the adult emerges from the escape hatch it simultaneously ecdyses from the pupal cuticle, characteristically leaving the pupal exuvium protruding from the exit hole in the leaf sheath (Figure 3).

Egg: typically dipteran in configuration: elongate, length about 0.5 mm, blunt at one end, tapering to a point at micropylar end. No respiratory horns visible. Color pinkish transparent. Observed in masses of 15 to 25 (Figure 4). Under



FIGURE 2: Third instars are found with the ambrosia fungus, a brown yeasty material, in the mesophyll layer of *Arundo donax*. Note the silken cocoons at center and upper left.



FIGURE 3: Pupal exuviae protruding from exit holes in the leaf sheath following emergence of the adults.

high magnification one can see within each egg yellowish vacuoles that are presumably yolk protein.

First instar: color pale sometimes pinkish, translucent, elongate fusiform in body shape; length 0.4–0.5 mm at eclosion. Two conspicuous orangish spots visible in abdomen at eclosion (Figure 5). These conspicuous spots may be the same vacuoles of yolk protein visible in the eggs. Soon after eclosion the material diffuses through the gut tract as the larva crawls away from the egg mass. Bolwig's organ, the subcuticular eyespots, is reddish and found between head capsule and prothorax (Figure 6). The cuticle is transparent and the head capsule, tentorium, and spiracles are nonsclerotized. The head capsule at this instar is almost as wide as the body segments. The antennae are orbicular with terminal nipple. The microverrucae of the cuticle are spinulate, particularly in the rows at the anterior margins of the body segments.

The first instar has a complement of haired papillae arrayed in number and position characteristic for

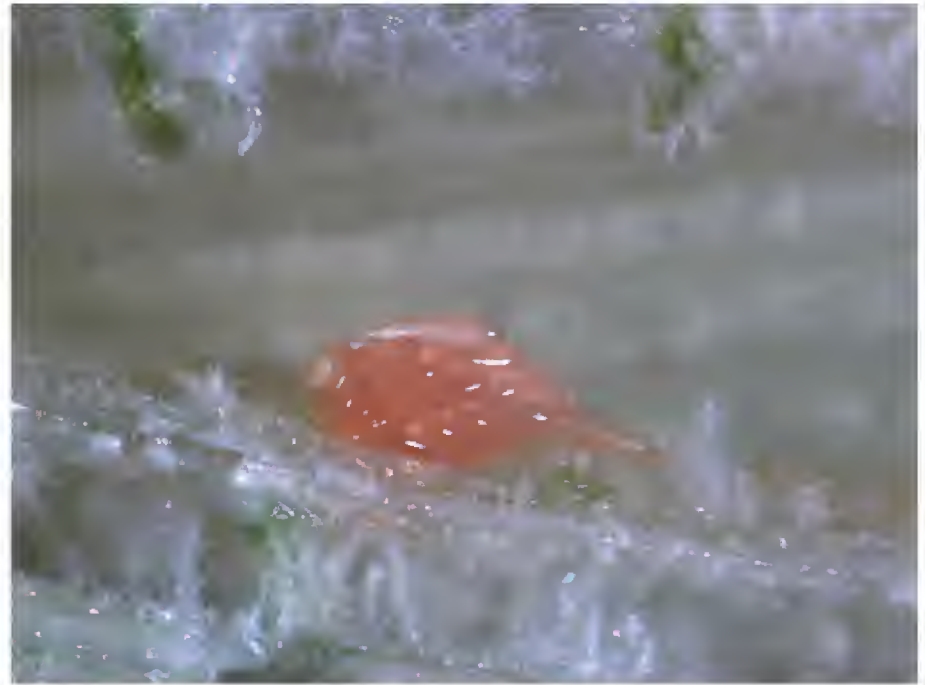


FIGURE 4: A clutch of eggs in a mesophyll cell of *Arundo donax*.



FIGURE 5: A recently hatched first instar. The orange spots are vacuoles of yolk carried over from the egg.

the Lasiopteridi [1]: six dorsals on each thoracic segment and first eight abdominal segments; a pair of pleural papillae on each side; and a pair of hind-ventrals on all postcephalic segments except penultimate and terminal segments. Penultimate segment has four ventrals and terminal segment with eight terminal haired papillae, the hairs 3–4 μm long, about twice as long as papillus on which they are seated. The eighth-abdominal segment has larger pleural papillae, the hairs of which are 4.5–5.3 μm long (Figure 7). The first instar does not have the sternal spatula nor does it have discernible sternal or lateral papillae. The body (Figure 8) lacks the long (30 μm) bristles and numerous spines reported in *L. arundinis* [27].

Second instar: color pale yellowish, length 1.0–1.75 mm ($n = 4$). Second instar larvae do not have the sternal spatula. Head capsule distinctly smaller in proportion to the body segments compared to the first instar and with antennae more elongate (Figure 9). The cuticle is transparent and with back-lighting under the compound microscope in each abdominal segment fat body is visible as three large darkened amorphous masses of tissue: one at middle and one on each side. Lateral



FIGURE 6: First instar, head and thoracic segments. The ovoid, red objects between the tentorial arms are Bolwig's organs. Note the rows of cuticular denticles.



FIGURE 8: The first instar does not have long mycangial bristles as reported in *Lasioptera arundinis*. Note that the head capsule is nearly as wide as the prothorax.



FIGURE 7: First instar, terminal abdominal segments showing haired papillae, the longest of which are those attendant to the spiracles.

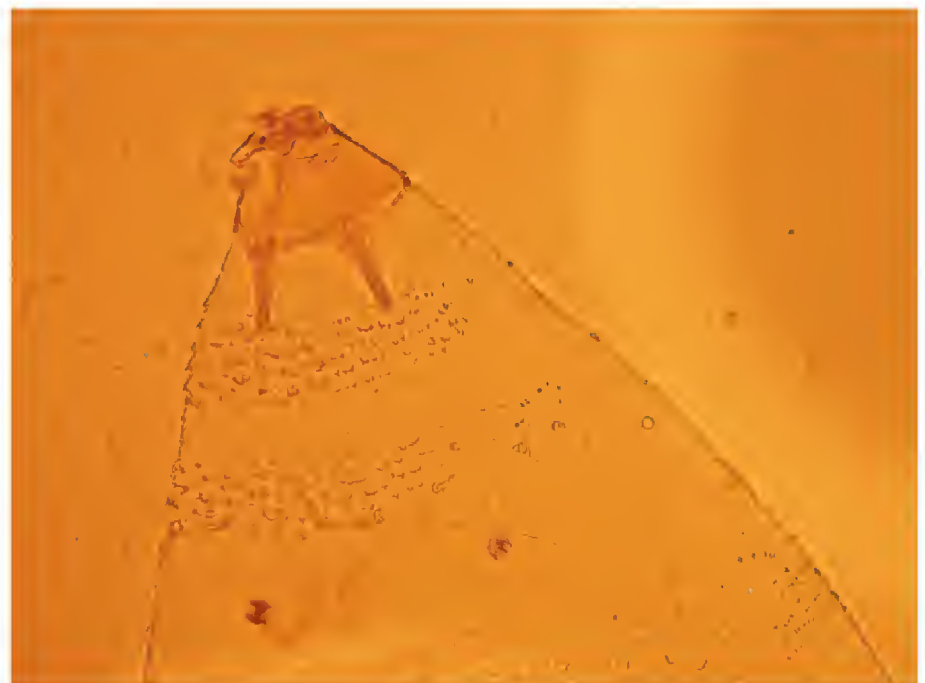


FIGURE 9: The head, neck, and prothorax (with spiracles) of the second instar. The spatula is absent. The dorsal and pleural papillae are setiform. The sternal and lateral papillae are absent.

papillae on thorax have 11–15 μm long bristles while those on eighth-abdominal segment are 16–19 μm long. Spiracles are present on prothorax and first eight abdominal segments.

Third instar: color orange in life fading to yellow in preserved specimens. Mean body length 3.06 ± 0.29 mm; range 2.66–3.71 mm ($n = 10$); width 0.57 ± 0.04 ; range: 0.51–0.65 mm ($n = 10$). Hemicephalic; body orthosomatic, asymmetrically fusiform, widest at mesothorax, somewhat dorso-ventrally compressed (Figure 10). In addition to the three thoracic segments and the nine abdominal segments, there is a pseudosegment, a neck or cervical segment between the head and prothorax. All body segments except neck segment and last two abdominal segments bulge laterally. Penultimate abdominal segment longest and ninth (terminal) segment shortest.

Spiracles of prothorax and eighth (penultimate)-abdominal segment prominent, turreted (Figure 11). Those on penultimate segment situated laterally in a plica (fold) anterior to

the posterior intersegmental suture. Spiracles of remaining segments situated dorsolaterally, and though sessile, annular, and inconspicuous, apparently functional because they are connected to trachea. The tracheae are darkened terminally where attached to peritreme, particularly on sixth and seventh abdominal segments.

Body without macropapillae or projections. Also lacking the microdentition seen in first instars. Rather the epidermal surface is minutely verrucose (cuticular verrucae of Mamaev and Krivosheina [14]) on sides and dorsum of all segments, whereas on the ventral surface the microverrucae are confluent and arrayed in transverse lines, presumably as traction ridges. The head is small, retractile, prognathous, the capsule lightly-sclerotized, somewhat conical, anchored with prominent exocephalic tentorial arms. A pair of two segmented (pseudosegmented *cf* Solinas et al. [28]) antennae are present, situated subapically, one on each side of mouthparts. Pedicel



FIGURE 10: The spatulate third instar larva. Note that the head capsule is comparatively minute.



FIGURE 11: A turreted spiracle on the penultimate abdominal segment.

short, not longer than wide; scape acuminate, fusiform, elongate, length about 3x width. In some images the large placoid sensilla is visible on each scape (Figure 12). The mouthparts are rudimentary and consist of a two-sided labrum, the minute peg-like maxillae, and tiny mandibles. Small, linear, and sclerotized, the latter are aligned parallel to one another and to the sagittal (vertical) axis of the cranium (Figure 14).

The haired papillae are reduced in number from the first instars (Figure 13). The terminal segment has eight stout bristles in two rows of four ranging in length from 10 to 22 μm , the ental pairs longer (mean $14.1 \pm 3.1 \mu\text{m}$, $n = 12$). Setiform dorsal papillae in a row of six are present on pro- and mesothorax with two on eighth abdominal segments between the spiracles. Their length varies from 22 to 42 μm (mean $32.9 \pm 5.5 \mu\text{m}$, $n = 25$). All thoracic and first eight abdominal segments have a pair of hind ventrals. Prothorax, mesothorax, and eighth abdominal segments with a pair of pleural setae on each side, remaining segments with one on each side. Outer pleural seta of prothorax longest at 38–50 μm



FIGURE 12: Head of the third instar. Arrow points to the placoid sensilla on the scape of the antenna.

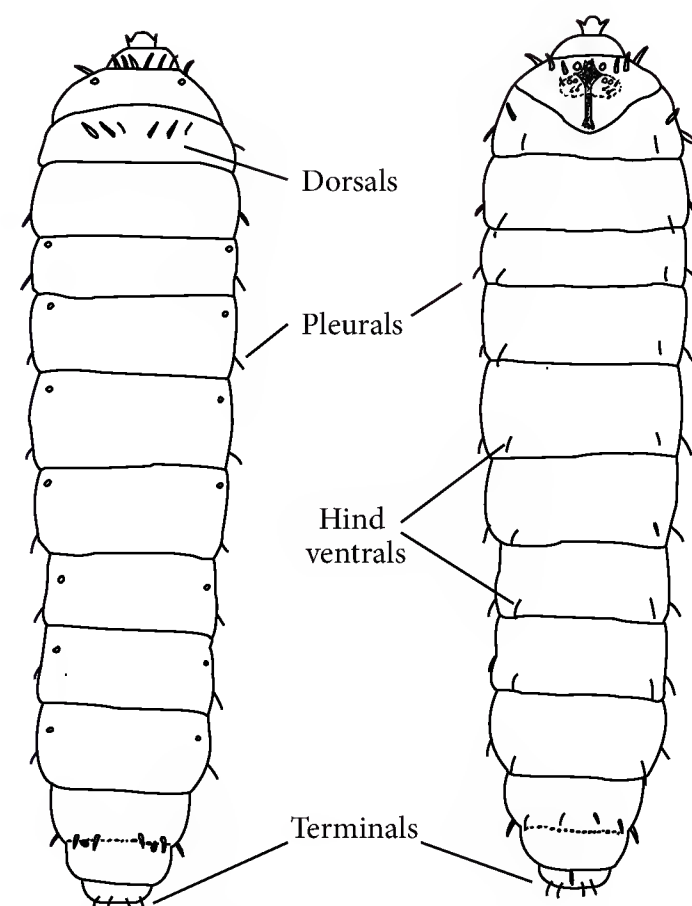


FIGURE 13: Diagrammatic position of setiform papillae on the third instar, dorsal on left, ventral on right.

(mean $41.1 \pm 5.5 \mu\text{m}$, $n = 10$), those on mesothorax next longest at 28–35 μm (32.5 ± 6.4 , $n = 11$) with those on the metathorax and abdominal segments shortest at 12–17 μm (24.7 ± 8.3 , $n = 9$). Hind ventrals are much smaller (mean $17.1 \pm 5.3 \mu\text{m}$, $n = 7$). Inner pleural papillae are haired on pro- and mesothorax; longer on prothorax (mean $32.5 \pm 3.8 \mu\text{m}$, $n = 4$) than on mesothorax (mean $22.3 \pm 4.2 \mu\text{m}$, $n = 7$) with those on eighth-abdominal segment even shorter at 18 μm . Metathorax and first seven abdominal segments are without dorsal or sternal haired papillae.

The number and position of setiform papillae in Möhn's [10] account of *Lasioptera arundinis* are somewhat problematic. He states that there are three pleural papillae on each side of the prothorax but that the third is often unhaired. We

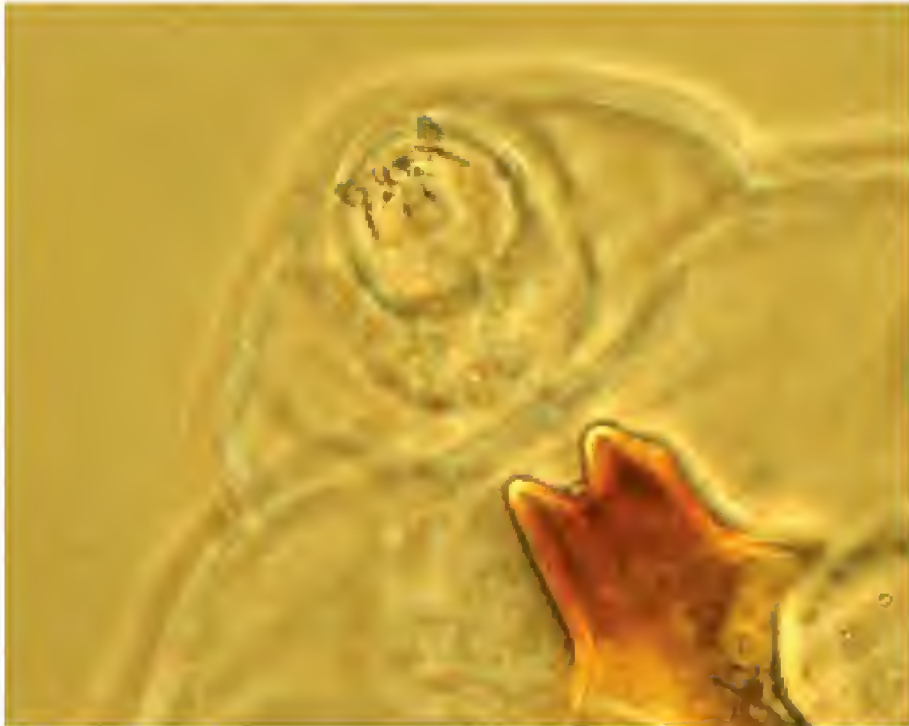


FIGURE 14: Ventral view of third instar head capsule showing rudimentary, paired mouthparts, and the three pronged spatula.



FIGURE 15: Setiform papillae flanking the spiracle on abdominal segment eight: one dorsal and two pleurals on each side.

find only two on *L. donacis*. Möhn's figure of the abdominal segments depict a single pleural seta; yet his text description states there are two, and that is the number that we find in *L. donacis* as well (Figure 15).

The ventral side of the prothorax has the prominent sclerotized armature referred to as the sternal spatula [29] or breast plate [30], length 0.37 ± 0.04 mm ($n = 8$). Seated on and protruding from the midline it is about ten times longer than greatest width and widest anteriorly where it is anchored to the body by sclerotized lateral apophyses just posterior to the terminus (Figures 14 and 16). The anterior projection is strongly sclerotized, protruding from the body and bearing three forwardly directed teeth: two broad lateral teeth with a smaller middle tooth between and somewhat entad of the lateral teeth. A cross section through the spatula reveals that it is actually hollow. The caudal end of the spatula is expanded into a rounded base, the manubrium, which is embedded deeply in the cuticle. Internally there is a large V-shaped mass

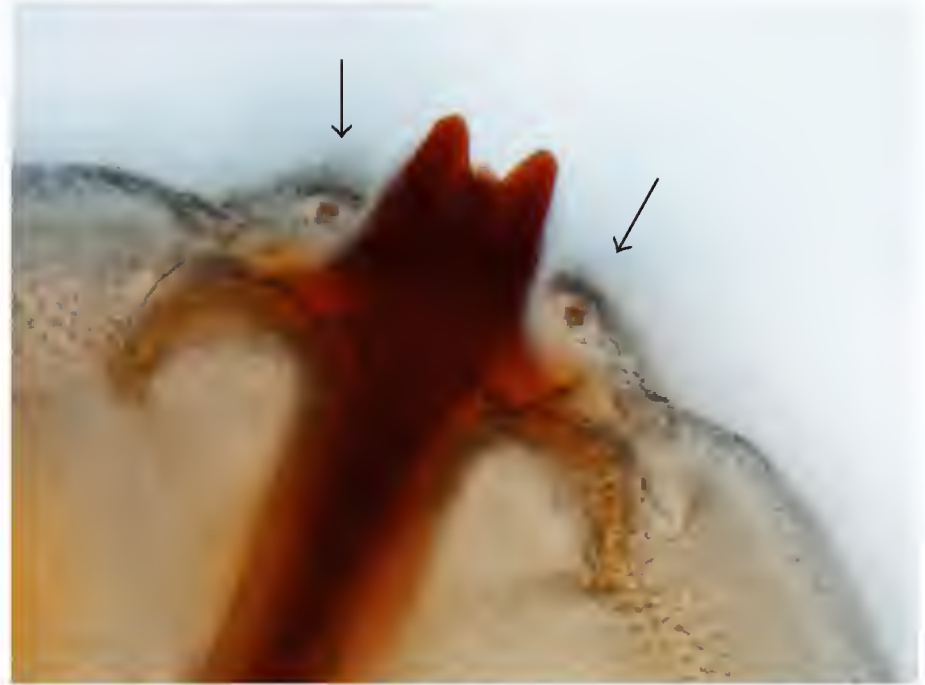


FIGURE 16: The three-pronged spatula is flanked by fleshy pads where the apodemes insert. The sternal papillae are seated between the spatula and the neck segment, one on each side (arrows).

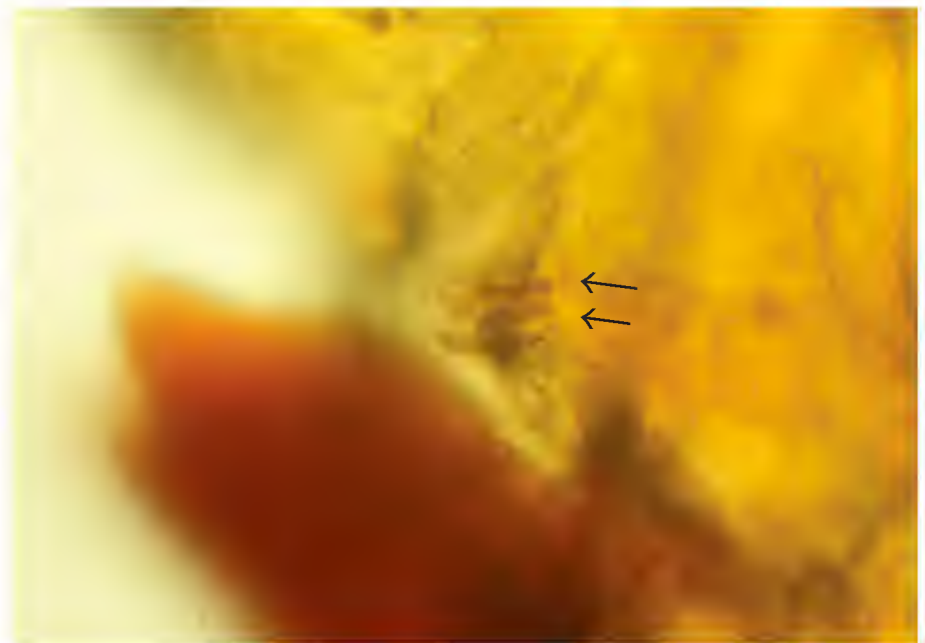


FIGURE 17: In profile the sternal papillae appear to have a pair of ducts (arrows) connecting to the paired sensory structures within its crater.

of muscle attached to the manubrium which inserts on the dorsum of the prothorax.

Attendant to each side of the anterior expansion of the spatula there is a fleshy pad bearing 3 + 2 lateral papillae. The three on the anterior margin are sclerotized and tubular or socket-shaped (Figure 18). The outermost of these bears a stout bristle, although in most specimens it is missing, presumably broken off from wear and tear. The two posterior, discal papillae are simple haired papillae (Figure 19). Located on the anterior portion of the prothorax ventrally, between the spatula and the head, there are two conspicuous crateriform structures, one on each side, referred to as the sternal papillae in the taxonomic literature although they are not papilliform (Figures 16 and 17).

Pupa: exarate, enclosed within a silken cocoon; capable of strong movement when disturbed. Body elongate, cylindrical; color off-white in early stages turning to orange later; wing pads black and with darkened patches of scales on the abdominal dorsum in fully mature, pharate specimens

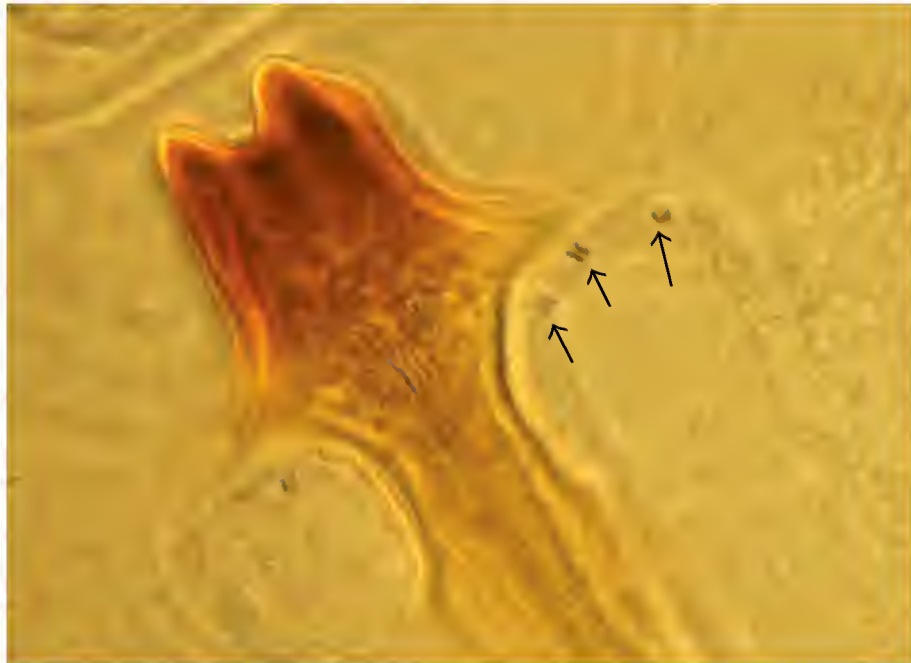


FIGURE 18: The fleshy pads attendant to the spatula have three sclerotized, socket-shaped papillae arrayed on their frontal margin (arrows).

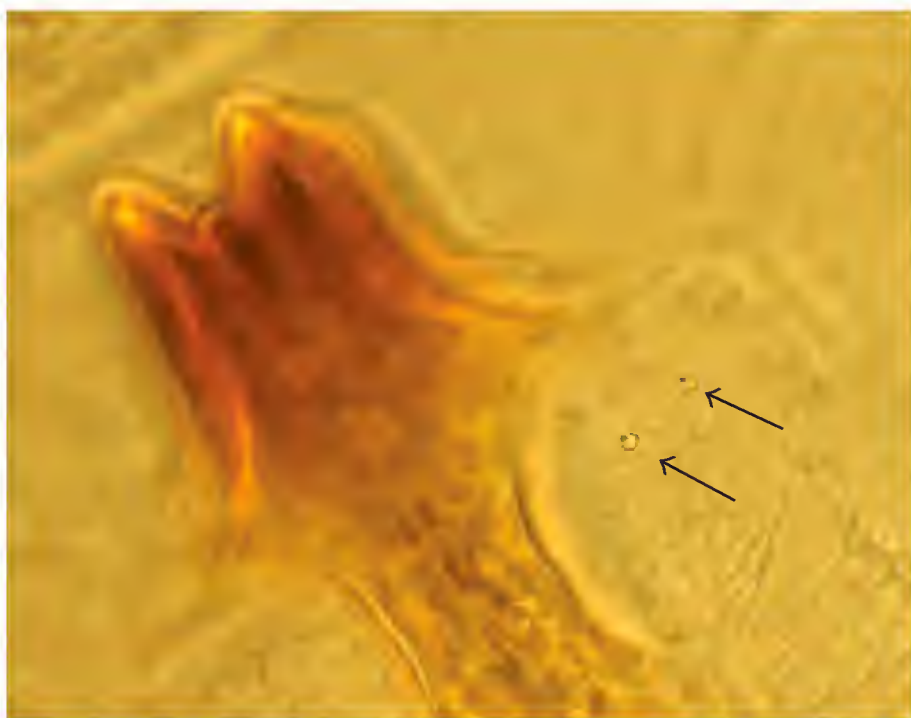


FIGURE 19: On the disc of each fleshy pad is a pair of haired papillae (arrows).

(Figures 20(a) and 20(b)). Abdominal segments without spines. Length: ranging from 2.2–3.0 mm (mean = 2.55 ± 0.24 mm, $n = 9$). Respiratory horns of thorax slender, tubular basally, filamentous apically. Leg sheaths nonoverlapping, unequal in length; metatarsi attaining abdominal terminus, mesotarsi nearly attaining abdominal segment VIII, protarsi attaining abdominal segment VI. Wing pads overlap metafemora. Beaded antennal sheathes overlap mesofemora and extend just past the base of the wing pads. Palp sheaths extend obliquely anterolaterad, reclining in the space between the eye and profemora. Frons without papillae, horns or ridges. Eyes entire, ocelli absent. Cephalic sclerite bearing a pair of prominent setae each inserted just behind the antennal base.

4. Discussion and Conclusions

The larvae of free-living cecidomyiids are variable with respect to the number and size of excrescences on the body

surface as illustrated by Peterson [30], Mamaev and Krivosheina [14], and Gagné [11–13]. Such excrescences are typically absent in the endophytic species, including *Lasioptera*. On the other hand, the form of the body, elongate, cylindrical, and bulging at the thorax (Figure 10), is typical of stem-boring or tunnel inhabiting insect larvae from which one can infer that its morphology facilitates movement within a confined space.

The sternal spatula is a characteristic feature of third instar cecidomyiid larvae. Milne [31] found that soil inhabiting species use the spatula to excavate the pupal chamber in the soil. Mamaev and Krivosheina [14] state that phytophagous species use the spatula to perforate or enlarge perforations in plant tissue. Among cecidomyiid species variation occurs primarily in the shape of the apex which can have one, two, or three prongs [14]. *Lasioptera donacis* has a three-pronged spatula. Among the 20 European species of *Lasioptera* larvae described by Möhn [10], only *L. arundinis* has a three-pronged spatula, the others being cloven with two prongs. A species from Australia, *L. uncinata* Gagné also has a three-pronged spatula, but with the middle prong the longest and it has only four lateral papillae (five in *L. donacis* and *L. arundinis*). Also the host for *L. uncinata* is a non-graminaceous plant, *Melaleuca* spp. (Myrtaceae) [17]. According to Gagné [1] none of the New World *Lasioptera* spp. have grasses as the host. *Lasioptera arundinis* is the species most similar in morphology and ecology to *L. donacis*. Because the former exploits an endophytic fungus in a closely related grass species, common reed, *Phragmites australis* (Cav) Trin., *L. donacis* and *L. arundinis* may be closely related species. Or it may be convergence that they share important morphological features in the third instar including a three-pronged spatula and five lateral papillae. The difference between the first instars of *L. donacis* and *L. arundinis* is the presence of long bristles and spines in the latter which relates to a difference in their ecology. According to Rohfritsch [27] *L. arundinis* lays its eggs outside of the plant under the leaf sheathes of the side shoots. The bristles and spines enable the first instar to carry the fungal conidia which are then disseminated into the plant as the larva feeds on and penetrates the cortical parenchyma of the stem. Because *L. donacis* females lay their eggs along with the conidia into preexisting holes in the leaf sheath surrounding the main stem, the larvae of the latter species do not require such bristles. Also, according to Rohfritsch [4] the first instar larva of *L. arundinis* uses its mandibles to perforate the lamella and enter the pith of the stem. The first instar of *L. donacis* has minute, rudimentary mandibles.

In spite of the reduction in the size of the mouthparts, cecidomyiid larvae, in particular those of the economically important phytophagous species, are noted for the damage inflicted on the host plant. In the case of the Hessian fly, *Mayetiola destructor* (Say), and the sorghum midge, *Contarinia sorghicola* (Coquillett), the damage is evidently caused by the injection of digestive fluids rather than by the physical damage by the tiny mandibles [16, 32]. Extraoral digestion is the norm for cecidomyiids whether phytophagous, mycetophagous, or even predaceous [14]. For that purpose the salivary glands are typically enlarged [32]. Our sections of

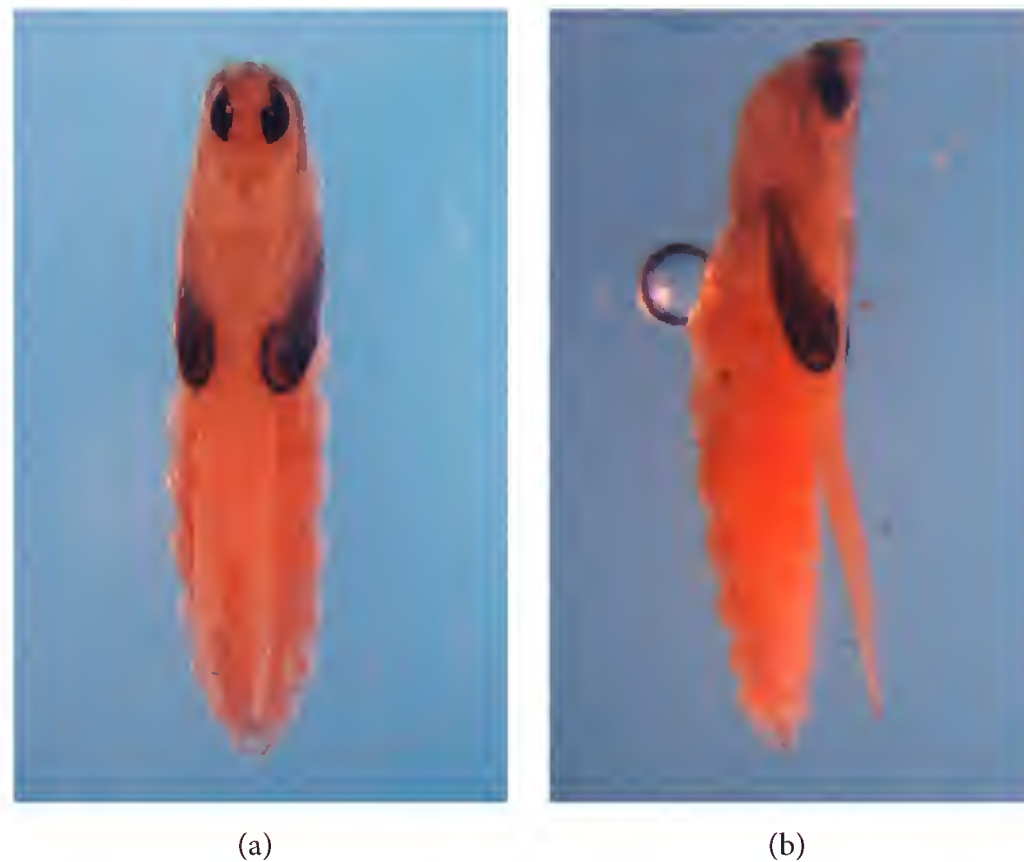


FIGURE 20: Late stage exarate pupa of *L. donacis*, ventral and side views.



FIGURE 21: A prepupal third instar makes a cocoon with silk from the labial gland.

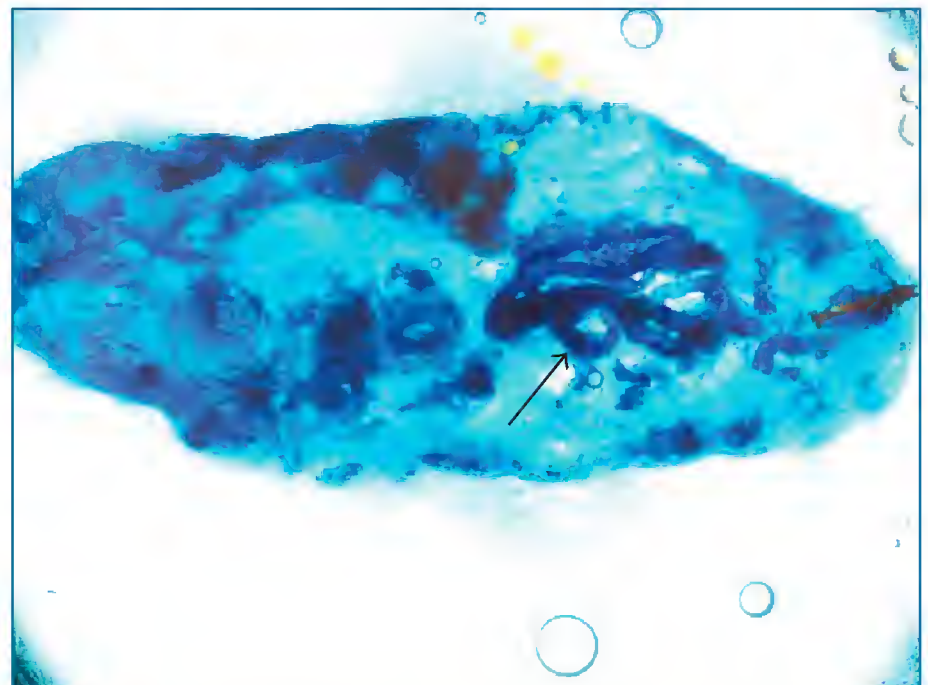


FIGURE 22: Longitudinal section of the late third instar shows the tubular salivary gland (arrow) associated with extraoral digestion and silk production.

L. donacis larvae show that the salivary gland dominates the internal thoracic space of the third instar (Figure 22). In cecidomyiids the glands have a dual function: producing digestive fluids during the feeding stage but also producing the silk for the cocoon (Figure 21) in the prepupal stage [33]. We presume that to be the case for *L. donacis* as well. Apart from having their heads directed at and into the fungal mycelium, we have not observed feeding directly in the *L. donacis* larvae, presumably because of their light aversion response. Our presumption of extraoral digestion is based on the rudimentary size of the mouthparts and the enlargement of the salivary glands, but also we have observed *vomit* when the larvae are placed on filter paper. The lack of accumulations of frass in the colonies, of which residue is typically a conspicuous aspect of insect tunnels, may be a consequence of the mainly liquid diet. Rohfritsch [2] describes feeding

by *L. arundinis* on the endophytic fungus and reports that in combination with extraoral digestion the larva uses the spatula to scrape and scratch the mycelium and then sucks in the solubilized predigested material. The juxtaposition of the spatula to the mouth opening in *L. donacis* suggests that the spatula serves as an extraoral masticating organ as well (Figure 23).

The number and distribution of the minute haired papillae of the first instar of *L. donacis* correspond to the chaetotaxy characteristic of the tribe Lasiopterini. In the later instars the number is reduced, especially on the abdomen, while those that persist have been transformed into sensory bristles. Whereas the socketed terminal bristles of the third instars are short and stiff (Figure 24), those on the thorax are long and flexile (Figure 25). In accordance with

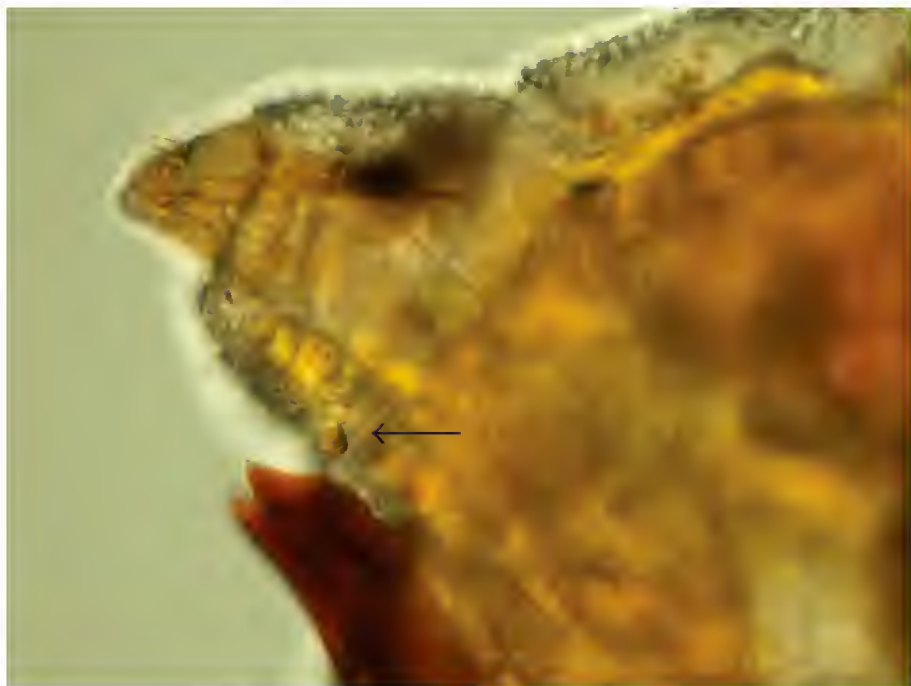


FIGURE 23: Lateral view of the prothoracic spatula. The head segment with antenna is to the far left. The head is seated in the collar segment within which the dark red Bolwig's organ can be seen (far right). On the prothorax between the spatula and the collar segment are the sternal papillae, one of which can be seen as a round, tan colored structure (arrow). When the collar segment inverts it brings the mouth into close juxtaposition to the spatula and the sternal papillae.

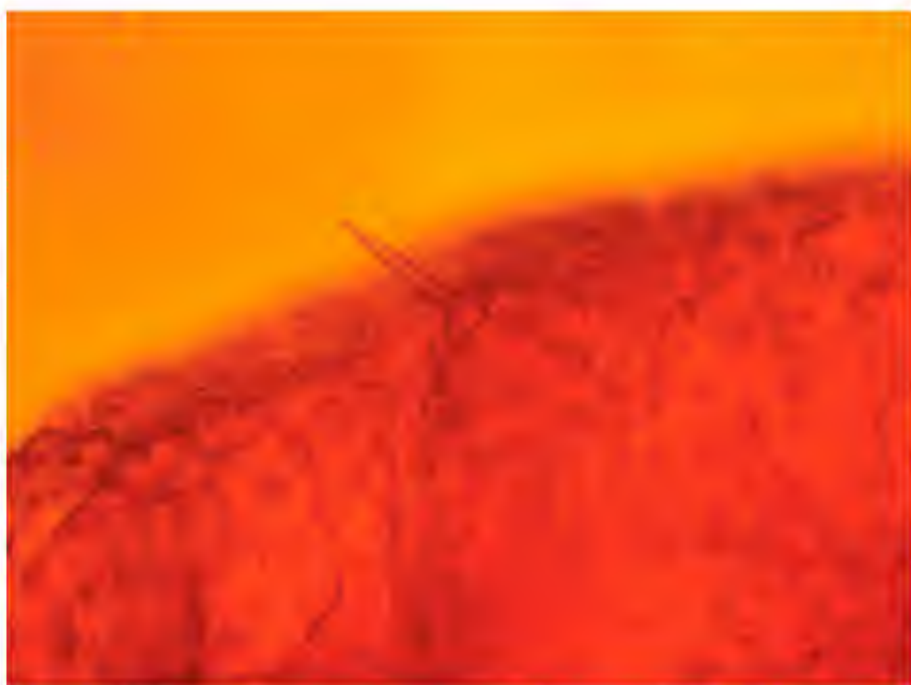


FIGURE 24: The terminal papillae of the posterior abdominal segments are stout socketed bristles and appear to be sensilla chaetica.

the nomenclature of Zacharuk and Shields [25] their structure suggests that the terminals are sensilla chaetica, which are tactile in function, while the thoracic bristles are sensilla trichodea, which are chemosensory as well as tactile. The dorsal bristles on the prothorax are arranged in a corona surrounding the head when it is retracted into the neck segment (Figure 26).

It strikes us that the role of the sensory organs in the endophytic species has been underappreciated. Bolwig's organ detects light [34] and is presumed to initiate escape and evasion behavior in the event the larvae is exposed. Skuhrava and Skuhravy [35] report that birds break into the cane to predate on the larvae. According to Solinas et al. [28] the gall-inhabiting cecidomyiid larvae have shorter antennae

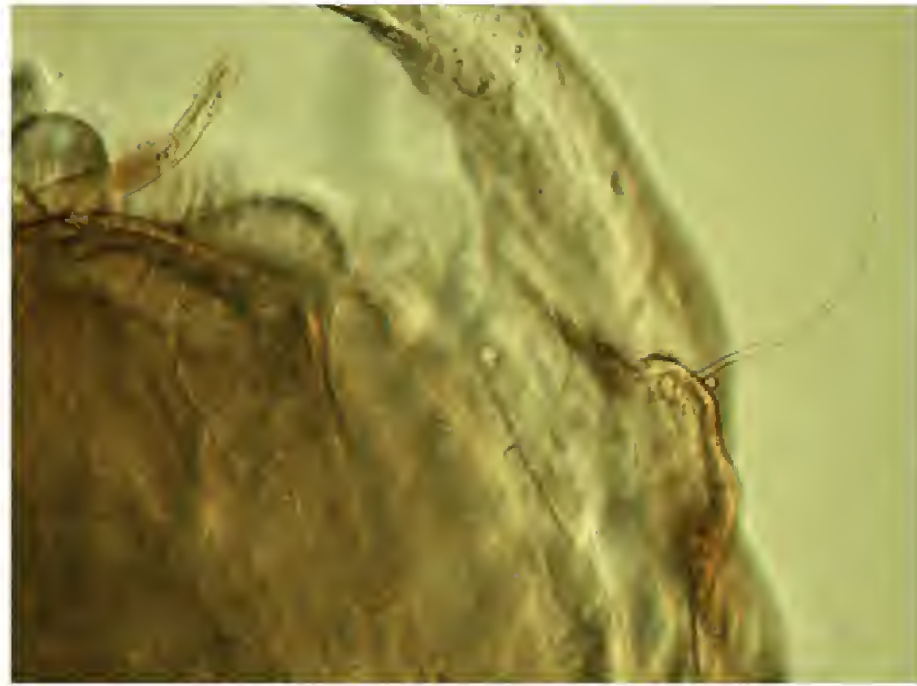


FIGURE 25: The pleural papillae on the prothorax have the form of flexile socketed setae, that is, sensilla trichodea.



FIGURE 26: Retracting the head into the neck segment juxtaposes the dorsal setae of the prothorax, three on each side ((a), (b), (c)) onto a fold collaring the head.

compared to the free living species and that is certainly the case with *L. donacis*. Whereas the antennae are small and the body is largely devoid of protuberances, the prothorax has conspicuous sensory papillae associated with the spatula. On either side of the frontal attachment of the spatula there is a fleshy pad which has the five lateral papillae. In most described species of *Lasioptera* there are four except in *L. arundinis* which also has five in a similar configuration to *L. donacis* as illustrated by Möhn [10]. Three are situated just behind the anterior margin and are sclerotized and tubular or socket-shaped. Behind them on the disc of the pad are two haired papillae. In the review of the sensory organs of larval insects by Zacharuk and Shields [25] there are no organs which are morphologically analogous to these structures. Being seated on the pads at the insertion of the spatula would suggest that their function may be proprioception related to the operation of the spatula in mastication. Among the postcephalic sensory organs proprioceptors such as

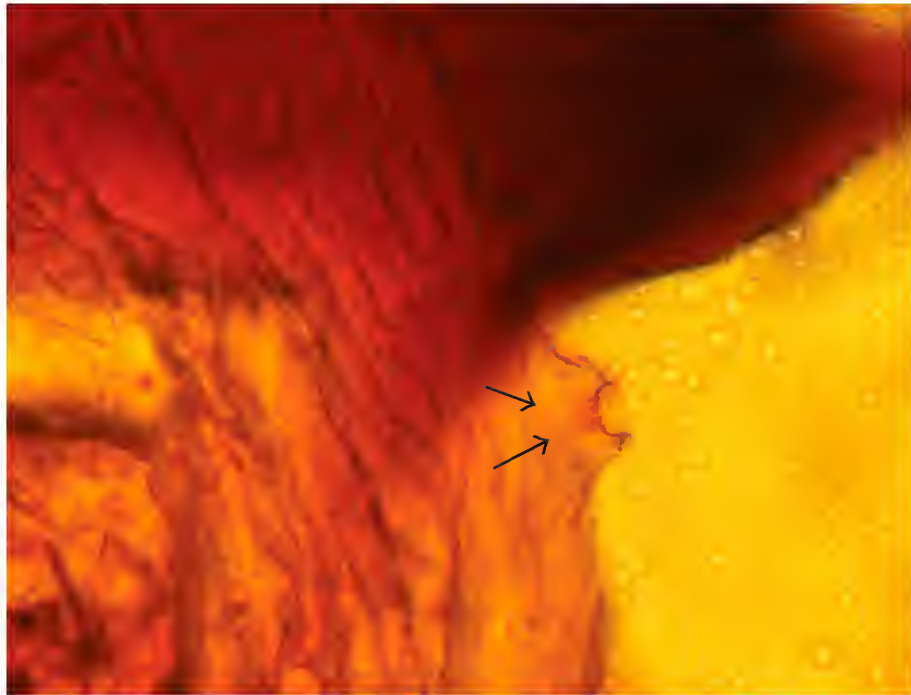


FIGURE 27: The sternal papillae in ectal view appear to be conical with paired structures internally (arrows), suggesting that they have a sensory function.



FIGURE 28: The paired structures within the sternal papillae are extrusible and may be sensory pegs (arrows).

the campaniform sensillae are common [36], but they are much smaller than these novel structures.

Even more conspicuous are the much larger sternal papillae, one on each side, seated on the ventral side of the prothorax between the spatula and the collar segment. In ectal view they appear to be conical with a pair of internal structures (Figure 27). In profile these structures appear to be connected to ducts (Figure 17). Moreover, the structures are eversible; that is, observed in living specimens they sometimes protrude from the central crater as pegs (Figure 28). This peg-in-pit arrangement in accordance with the system of Zacharuk and Shields [25] would be sensilla ampullacea which are chemosensory. The number of pegs varies among larval taxa, as many as seven in some Staphylinidae (Coleoptera) [37]. If they are two-pegged sensilla ampullacea the sternal papillae on the prothorax of *L. donacis* are perhaps gustatory in function, that is, an adaptation to extraoral digestion. The juxtaposition of these sensillae near the spatula would

perhaps allow the larvae to “taste” the macerated mycelial mass as it feeds. They may thus be functionally analogous to the papilla sensilla found on the terminal organs of the head segment of house fly larva [38]. In the latter study the two pegs are referenced as “dendrites” exposed to the exterior and functioning as taste organs.

The reliance on an endophytic fungus for its food resource complicates but does not eliminate *Lasioptera donacis* as a candidate for biocontrol of the invasive weed, *Arundo donax*. However one can expect that a deeper understanding of the tritrophic relationships among the plant, the fungus, and the insect will be required before a successful program can be implemented.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Diversity and Distribution of Aquatic Insects in Streams of the Mae Klong Watershed, Western Thailand

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The distribution and diversity of aquatic insects and water quality variables were studied among three streams of the Mae Klong Watershed. In each stream, two sites were sampled. Aquatic insects and water quality variables were randomly sampled seven times in February, May, September, and December 2010 and in January, April, and May 2011. Overall, 11,153 individuals belonging to 64 families and nine orders were examined. Among the aquatic insects collected from the three streams, the order Trichoptera was most diverse in number of individuals, followed by Ephemeroptera, Hemiptera, Odonata, Coleoptera, Diptera, Plecoptera, Megaloptera, and Lepidoptera. The highest Shannon index of diversity of 2.934 and 3.2 was recorded in Huai Kayeng stream and the lowest was in Huai Pakkok stream (2.68 and 2.62). The high diversity of insect fauna in streams is an indication of larger microhabitat diversity and better water quality conditions prevailing in the streams. The evenness value was recorded as high in most sites. The high species diversity and evenness in almost all sites indicated good water quality.

1. Introduction

In lotic environments, aquatic insects are important elements in the ecological dynamics [1], playing an important role in the cycle of materials and in trophic transfers [2–5]. The understanding of distribution patterns in communities is one of the main aims in ecology [6]. A multiplicity of factors regulates the occurrence and the distribution of aquatic insects, the most important being the current (velocity), temperature, altitude, season, total suspended solids, and vegetation [7]. Other factors which affect the occurrence of these benthic fauna include substrates, pH, dissolved oxygen, availability of food, turbidity, conductivity, and competition [8]. Changes of these environmental factors in streams can be used in biomonitoring and degraded aquatic environments [9, 10]. Thus, the nature of this distribution provides an initial insight into the types of ecological processes that regulate populations and assemblages. For example, the distribution of aquatic insects among stream habitats reflects, to some degree, the distribution of benthic resources (e.g., food, oxygen, and predators) and provides information about how communities might respond to changes in environmental

parameters such as increased sedimentation and changes in flow [11].

In this work, three streams in the Mae Klong Watershed were selected. These streams are used for domestic activities including drinking, cooking, bathing, and fisheries. It is therefore important to preserve these water resources and maintain the biotic integrity of these ecosystems. Such management requires basic knowledge such as the distribution of the aquatic communities among stream habitats. The aim of this study was to describe the composition of aquatic insects at different stations of the studied streams. The identification of species and their distribution patterns provide more information for monitoring and conserving these ecosystems.

2. Materials and Methods

2.1. Study Area. The Mae Klong Watershed is the most important watershed in western Thailand. The upstream watershed area consists of two main rivers, namely, Khwae Noi and Khwae Yai. The rivers run into the Khao Laem and Srinagarind Dam located in the upper region of Mae Klong

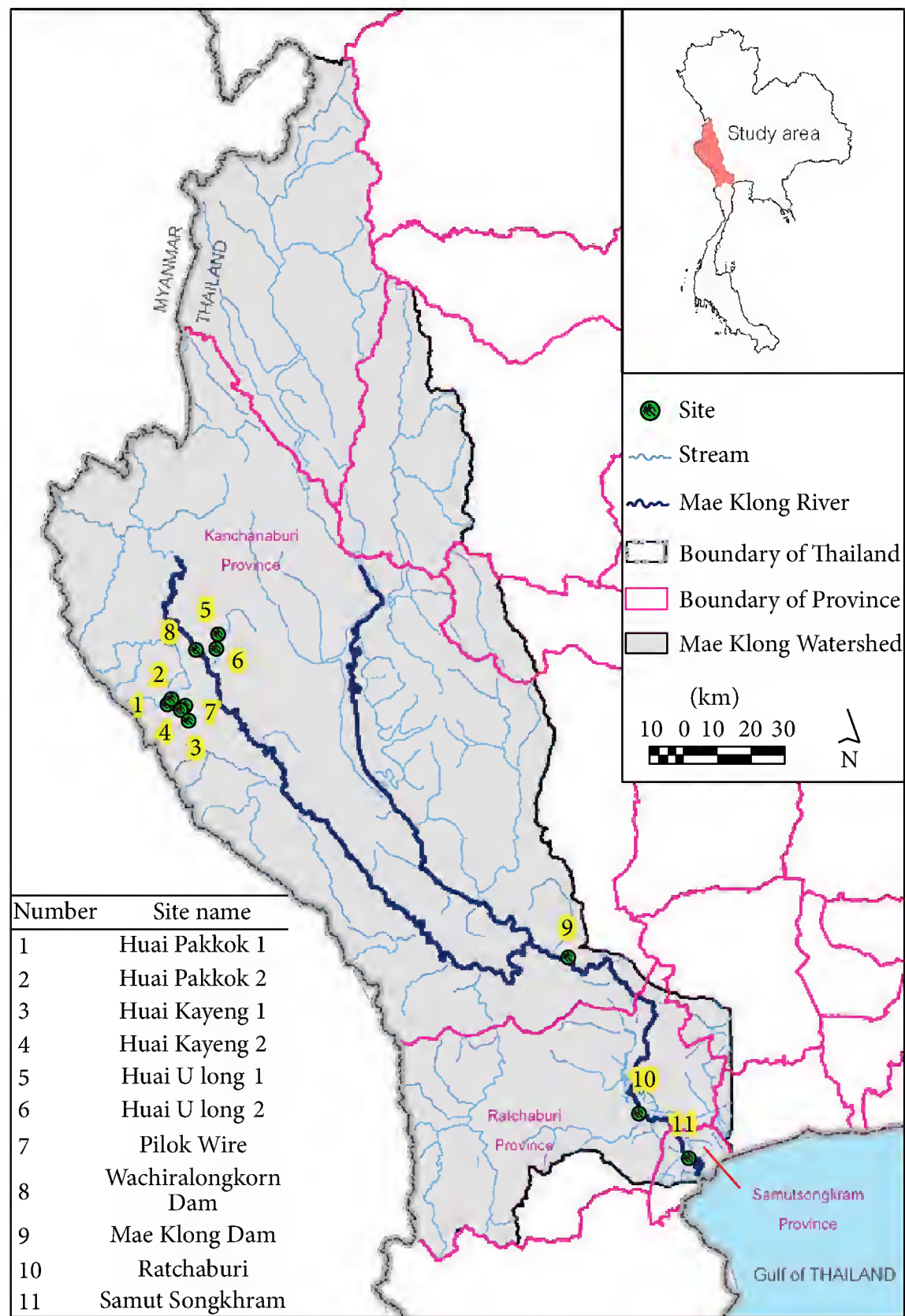


FIGURE 1: Map of Mae Klong Watershed showing the sampling sites, namely, Huai Pakkok, PK1 = 1 and PK2 = 2; Huai Kayeng, KY1 = 3 and KY2 = 4; and Huai U Long, UL1 = 5 and UL2 = 6.

Watershed. The rivers joint in Kanchanaburi Province, which is considered downstream, flow through Ratchaburi Province and enter the Gulf of Thailand in Samut Songkhram Province. Six sampling sites in three streams (upper and lower in each stream) were chosen in this study. These streams were in the upstream section of Khwae Noi River before flowing into Khao Laem Dam (Figure 1). These three streams are, namely, Huai Pakkok, PK1 and PK2; Huai Kayeng, KY1 and KY2; and Huai U Long, UL1 and UL2.

2.2. Sampling and Identification of Aquatic Insects. To determine the distribution of aquatic insect taxa, six sampling sites in three streams (upper and lower in each stream) were chosen in this study. Seven samplings were performed in February, May, September, and December 2010 and in January, April, and May 2011. Aquatic insects were collected using aquatic D-frame aquatic kick net (30 × 30 cm frame,

250 μ m mesh). At each sampling site, a stretch of approximately 50 m was chosen for collection of samples from the three target habitats: riparian vegetation, leaf litter, and low gradient riffles and pools. The sampling time at each habitat was 3 min. In each sampling period, three replicate samples were collected at each station and placed in white trays for sorting. The content of each sample was transferred into properly labelled plastic containers, preserved in 80% ethanol, and taken back to the laboratory for analysis. In the laboratory, aquatic insects were sorted and identified to the family level using taxonomic keys [12–14]. All the sorted samples were kept in properly labelled vials containing 80% ethanol.

2.3. Environmental Variables. Three replicates of selected physicochemical water quality parameters were recorded directly at the sampling sites including pH, air temperature

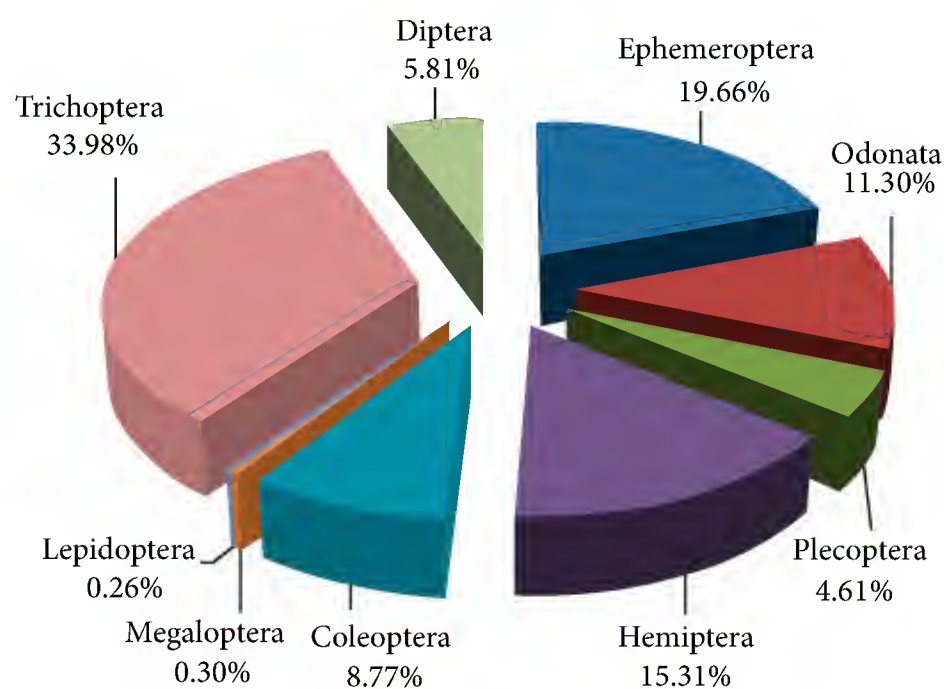


FIGURE 2: Composition of aquatic insect orders of the three streams in the Mae Klong Watershed during a period of seven months.

(AT), water temperature (WT), dissolved oxygen (DO), total dissolved solid (TDS), and electrical conductivity (EC). Water samples from each collecting period were stored in polyethylene bottles (500 mL). Ammonia-nitrogen ($\text{NH}_3\text{-N}$), orthophosphate (PO_4^{3-}), nitrate-nitrogen ($\text{NO}_3\text{-N}$), sulfate (SO_4^{2-}), and turbidity (TUB) were determined in accordance with the standard method procedures (APHA et al., 1992) [15]. Alkalinity (ALK) was measured by titration.

2.4. Data Analyses. The aquatic insect abundance and taxonomic richness (S) were estimated for each sample. Ecological indices, including the Shannon-Wiener diversity (H'), Simpson's diversity index (D), and Evenness (E) indices, were determined for each sampling site [16]. A principle component analysis (PCA) was performed using environmental variables to determine the abiotic typology of sampling stations. This analysis was performed with the matrix consisting of 42 samples (6 stations $\times$ 7 campaigns) and 12 environmental variables. Analyses were conducted using PC-ORD.

3. Results and Discussion

A total of 11,153 individuals of aquatic insects representing 64 families from 9 orders were collected and identified from three streams in February, May, September, and December 2010 and in January, April, and May 2011. Table 1 and Figure 2 show the overall composition and distribution of aquatic insect communities in the three streams. More aquatic insects were recorded in Huai Pakkok (PK1 and PK2) (2,054 and 2,726 individuals) than in Huai Kayeng (KY1 and KY2) (2,202 and 1,234 individuals) and Huai U Long (UL1 and UL2) (969 and 1,968 individuals). However, the total number of individuals recorded in the three streams was significantly different (One-Sample Test = 7.022, $P < 0.05$). Trichoptera (3,790 individuals; 33.98% of total abundance) was the most dominant order with the highest number of individuals in the three streams. It was followed by Ephemeroptera

(2,193 individuals; 19.66% of total abundance), Hemiptera (1,707 individuals; 15.31% of total abundance), Odonata (1,260 individuals; 11.30% of total abundance), Coleoptera (978 individuals; 8.77% of total abundance), Diptera (648 individuals; 5.81% of total abundance), Plecoptera (514 individuals; 4.61% of total abundance), Megaloptera (34 individuals; 0.30% of total abundance), and Lepidoptera (29 individuals; 0.26% of total abundance) (Figure 2; Table 1). The aquatic insects of Huai Pakkok stream (PK1 and PK2) constituted 50 families and 46 families, while 49 families and 46 families were recorded in Huai Kayeng stream (KY1 and KY2). The aquatic insects recorded from Huai U Long stream (UL1 and UL2) were represented by 41 and 49 families, respectively (Table 1).

Table 1 showed the species diversity indices. The highest Shannon index of diversity of 2.934 and 3.2 was recorded in Huai Kayeng stream (KY1 and KY2) and the lowest was in Huai Pakkok stream (HK1 and HK2) (2.68 and 2.62), indicating the presence of a higher diversity of aquatic insects in lotic ecosystems. The diversity of insects in aquatic ecosystems tends to increase with increased nutrients and these optimum environmental conditions favour their abundance in this habitat [17]. The high diversity of insect fauna in streams is an indication of larger microhabitat diversity and better water quality conditions prevailing in the streams (Table 2) [18]. Their abundance has been associated with the presence of high food quality, stable water flow, and stable substrata common in these habitats [17].

The evenness value in the present study was recorded as high in almost all the sites, indicating a relatively even distribution of taxa in the stream. The highest species diversity and evenness in almost all the sites are an indication of good water quality [18]. The high scores of diversity indices, such as those of the Shannon-Wiener index and Simpson's index, indicate that clean or unpolluted rivers support more diverse taxa, thus making them useful for detecting organic pollution [19]. Higher numbers of taxa (family) collected from a habitat imply a richer community that usually lives in a healthier environment. Based on the scores, all streams in the Mae Klong Watershed supported relatively rich aquatic insect fauna, but their composition and abundance were significantly different between rivers.

The differences in the physical habitat and hydrological conditions of streams could contribute to the observed dissimilarities in the aquatic insect compositions. This may be due to the multiplicity of microhabitats along with a combination of several other environmental factors that varied between streams [20]. Usually, similar richness of aquatic insects is recorded from streams with similar habitat structures, stream geomorphologies, and hydrological conditions [21].

By composition, Trichoptera and the Ephemeroptera dominated the study sites, accounting for almost 54% of all the total individuals that were sampled at the three streams. Heptageniidae (Ephemeroptera) and Hydropsychidae (Trichoptera) were found in all sampling sites because they are able to colonize waters with low oxygen concentration. Similar low numbers of Plecoptera in tropical waters have been reported [22, 23].

TABLE 1: The composition and total abundance of aquatic insect communities in Mae Klong Watershed.

Taxa	Abbv.	PK1	PK2	KY1	KY2	UL1	UL2
<i>Ephemeroptera</i>							
Pothamanthidae	Potha	1	1		1	1	7
Oligoneuriidae	Oligo		2	1			1
Caenidae	Caeni	5	1	3	4	1	7
Heptageniidae	Hepta	88	266	91	110	79	113
Leptophlebiidae	Leptp	247	218	58	40	65	76
Ephemerellidae	Ephem	20	97	17	50	20	94
Neoephemeridae	Neoep		1	6	8		
Ephemeridae	Ephee	2			9		5
Baetidae	Baeti	57	118	75	41	33	51
Prosopistomatidae	Proso				2		
<i>Odonata</i>							
Lestidae	Lesti		26	8			
Chlorocyphidae	Chlor	24	8	6	15	3	4
Protoneuridae	Proto	53	61	58	9	9	8
Gomphidae	Gomph	43	33	15	59	20	37
Libellulidae	Libel	20	26	16	9	4	19
Euphaeidae	Eupha	29	14	31	34	14	28
Corduliidae	Cordu	42	31	68	20	12	32
Aeshnidae	Aeshn		1			1	1
Platycnemididae	Platy	12	1	1	3	3	2
Coenagrionidae	Coena	62	39	53	24	8	
Calopterygidae	Calop	16	20	46	4	13	2
<i>Plecoptera</i>							
Perlidae	Perli	60	99	63	39	75	130
Peltoperlidae	Pelto	3	22			21	1
Nemouridae	Nemou	1					
<i>Hemiptera</i>							
Aphelocheiridae	Aphel	13	15	32	33	4	28
Gerridae	Gerri	69	128	50	52	24	40
Veliidae	Velii	19	10	23	13	3	16
Nepidae	Nepid	13	16	15	12	1	4
Hydrometridae	Hydrm	1	1				2
Helotrephidae	Helot	24	17	12	1	9	10
Naucoridae	Nauco	125	289	171	52	63	262
Pleidae	Pleid	13	1	2		5	
Notonectidae	Noton	6	2				
Micronectidae	Micro				1		5
<i>Coleoptera</i>							
Noteridae	Noter			1			
Hydrophilidae	Hydrp	12	8	8	10	19	3
Hydraenidae	Hydra	2	1	1			4
Gyrinidae	Gyrin	17	12	21	19	4	75
Elmidae	Elmid	79	29	91	46	299	50
Psephenidae	Pseph	23	9	26	20	9	12
Dytiscidae	Dytis			10	41	9	3
Scirtidae	Scirt			5			
<i>Megaloptera</i>							
Corydalidae	Coryd	6	6	10		3	9
<i>Lepidoptera</i>							
Pyralidae	Pyral	2	5	4	3		15

TABLE 1: Continued.

Taxa	Abbv.	PK1	PK2	KY1	KY2	UL1	UL2
<i>Trichoptera</i>							
Polycentropodidae	Polyc	2					
Hydroptilidae	Hydrt	1					1
Odontoceridae	Odont	1		30	8	1	52
Leptoceridae	Lepto	6		4	5	1	8
Calamoceratidae	Calam	6	8	76	145	26	60
Hydropsychidae	Hydrs	727	809	522	130	96	578
Helicopsychidae	Helic		2	324	91		
Philopotamidae	Philo	4	2	37	13		2
Lepidostomatidae	Lepid			1	2		5
Goeridae	Goeri			3			1
<i>Diptera</i>							
Athericidae	Ather	2		1	1		5
Tipulidae	Tipul	12	16	3	4	3	15
Simuliidae	Simul	21	195	34	5	3	23
Tabanidae	Taban	4		6	2	1	5
Ceratopogonidae	Cerat	1		1	1		7
Stratiomyidae	Strat	5					
Culicidae	Culic		3		3	1	
Psychodidae	Psych	6	2				
Sciomyzidae	Sciom					1	
Chironomidae	Chiro	47	55	62	40	2	50
Abundance		2,054	2,726	2,202	1,234	969	1,968
Richness		50	46	49	46	41	49
Evenness		0.685	0.684	0.754	0.836	0.712	0.712
Shannon-Wiener diversity		2.68	2.62	2.934	3.2	2.643	2.771
Simson's diversity index		0.8461	0.8709	0.9031	0.9441	0.8683	0.8775

TABLE 2: Mean values (±SD) of environmental variables in each sampling site.

Parameter/station	PK1	PK2	KY1	KY2	UL1	UL2
AT	31.35 ± 3.03	33.73 ± 1.73	31.87 ± 2.66	30.04 ± 4.36	30.57 ± 1.86	29.97 ± 3.68
WT	28.45 ± 3.02	29.82 ± 2.67	29.03 ± 2.17	28.58 ± 2.57	27.83 ± 1.15	28.11 ± 2.72
pH	8.05 ± 0.85	8.15 ± 0.9	8.15 ± 0.59	7.99 ± 0.99	8.43 ± 0.64	8.19 ± 0.53
DO	4.16 ± 2.08	4.45 ± 1.98	3.79 ± 1.63	5.0 ± 1.67	3.86 ± 0.65	6.35 ± 2.51
EC	74.08 ± 21.95	104.1 ± 30.72	275.38 ± 111.73	325.49 ± 91.15	119.68 ± 16.94	158.62 ± 69.67
TDS	37.02 ± 10.56	55.03 ± 13.5	135.44 ± 55	163.15 ± 43.27	58.3 ± 15.22	92.92 ± 33.39
TUB	3.33 ± 1.37	3.57 ± 3.36	9 ± 16.67	12.43 ± 24.53	5.0 ± 2.65	5.17 ± 4.96
ALK	58.33 ± 22.32	70 ± 20.40	106.67 ± 35	170.71 ± 33.76	132 ± 24.98	136.17 ± 24.33
NH ₃ -N	0.18 ± 0.18	0.14 ± 0.16	0.24 ± 0.19	0.22 ± 0.18	0.19 ± 0.19	0.18 ± 0.16
PO ₄ ³⁻	0.13 ± 0.06	0.09 ± 0.06	0.11 ± 0.06	0.07 ± 0.05	0.09 ± 0.08	0.14 ± 0.22
NO ₃ -N	1.35 ± 0.42	1.57 ± 0.24	1.52 ± 0.29	1.87 ± 0.81	1.47 ± 0.06	1.45 ± 0.16
SO ₄ ²⁻	1.2 ± 6	2 ± 10	2.2 ± 11	2.33 ± 14	5 ± 15	1.83 ± 11

PCA ordination for data of aquatic insects can be separated into two groups (Figure 3). The first group was located in the Huai Pakkok stream (PK1 and PK2) and the second group was located in the Huai Kayeng stream (KY1 and KY2) and the Huai U Long stream (UL1 and UL2). PCA analysis

revealed a correlation between the aquatic insect family and water quality (Figure 3). Aquatic insects in families Baetidae, Heptageniidae, Protoneuridae, Gerridae, Helotrephidae, Notonectidae, Nepidae, Leptoceridae, and Simuliidae were related to the concentration of orthophosphate and water

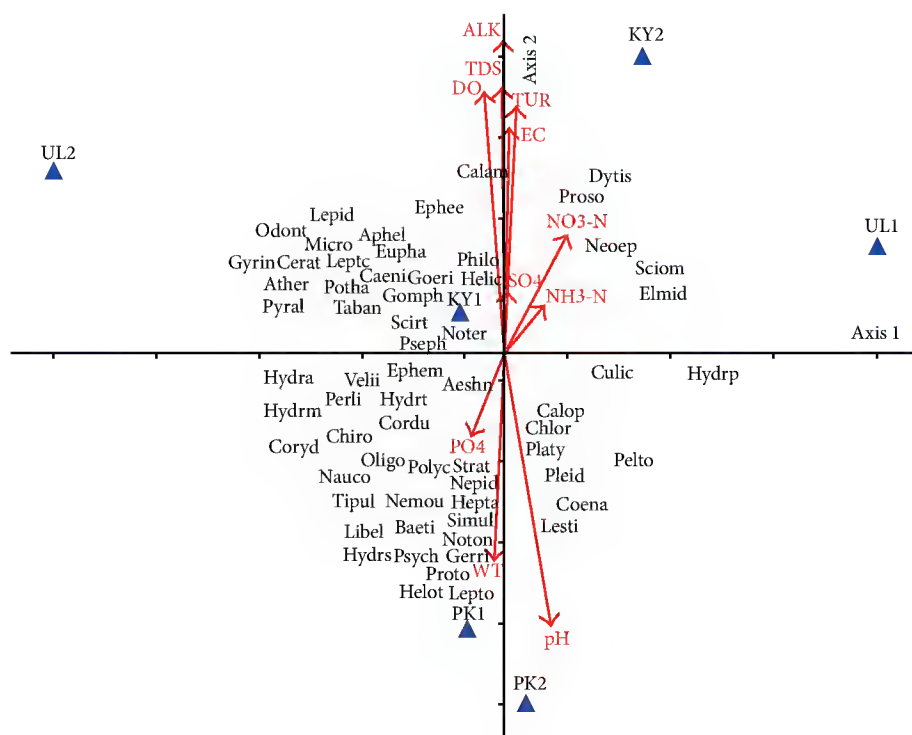


FIGURE 3: Biplot of the PCA ordination diagram for the data set between aquatic insect taxa, sampling sites, and environmental variables in the three streams. Abbreviations of taxa are shown in Table 1.

temperature. Aquatic insects in families Calopterygidae, Chlorocyphidae, Coenagrionidae, Lestidae, Platycnemididae, Peltoperlidae, Pleidae, Culicidae, and Hydropsychidae had relationships with pH of water. Water quality variables such as alkalinity, total dissolved solids, dissolved oxygen, turbidity, electrical conductivity, sulfate, nitrate-nitrogen, and ammonia-nitrogen affected aquatic insect families Neophemeridae, Ephemeridae, Elmidae, Dytiscidae, Calamoceratidae, Helicopsychidae, and Philopotamidae.

4. Conclusions

The results obtained in the present study indicate that, of the aquatic insects collected from the three streams, the order Trichoptera was most diverse in number of individuals, followed by Ephemeroptera, Hemiptera, Odonata, Coleoptera, Diptera, Plecoptera, Megaloptera, and Lepidoptera. The highest Shannon index of diversity was recorded in Huai Kayeng stream and the lowest was in Huai Pakkok stream. The evenness value was recorded as high in almost all sites. PCA analysis can expose the correlation between aquatic insect family and water quality with water temperature, orthophosphate alkalinity, total dissolved solids, dissolved oxygen, turbidity, electrical conductivity, sulfate, nitrate-nitrogen, and ammonia-nitrogen.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Broad Protein Spectrum in Stored Pollen of Three Stingless Bees from the Chaco Dry Forest in South America (Hymenoptera, Apidae, Meliponini) and Its Ecological Implications

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Protein content of pollen stored by three meliponine species was variable from 9.78% (less than half the value considered as optimal to brood development in *Apis mellifera*) in type *Capparis tweediana*-*C. speciosa* to more than 26% in type *Maytenus vitis-idaea* and some *Prosopis* samples. This pollen of low protein value was occasionally foraged (only six out of 75 masses analyzed of *G. argentina*, but none in 86 masses of *T. fiebrigi* or in ten of *M. orbignyi*). However, it is likely that amino acid deficiencies of certain pollens are compensated by randomly foraging on a broad spectrum of pollen plants. The large amounts of pollen stored in their nests might also be important in compensating these deficiencies. The only sample studied for *M. orbignyi* showed a protein value greater than the one required for *A. mellifera* and was dominated by types *Acacia praecox* and *Prosopis*. As this species also prefers *Solanum* and other protein-rich pollen, more samples would need to be analyzed to establish whether protein requirements are high for this *Melipona* species. Pollen showing the highest protein content (>26%) belonged to highly nectariferous plants well represented in meliponine and *Apis* honey such as *Prosopis*, *Maytenus*, and *Ziziphus*.

1. Introduction

The stingless bees (Apidae: Meliponini) are eusocial and they build perennial nests with hundreds to thousands of individuals and high reproductive rate [1, 2]. To maintain their colonies, high amounts of pollen and nectar are foraged from flowers, the former stored as pollen masses and the latter as honey in pots made of cerumen. Pollen pots are located mainly surrounding the brood area where young individuals are growing [3]. Pollen stored in nests is chemically and biochemically different from fresh pollen from anthers or pollen loads from the same plant species due to regurgitated liquids incorporated during packing [4–7]. Mutualistic bacteria were found in stored pollen and honey of nine *Apis* species, *Melipona fasciata panamica* Cockerell, *Melipona beecheii* Bennett, *Meliponula bocandei* (Spinola), and *Trigona* producing enzymes that may facilitate

storage and/or digestion by bees and secrete antibiotics and fatty acids to inhibit microorganisms that cause spoilage of stored food [8, 9]. During periods of general food shortage, *Melipona* species show conservative trends eating young larvae and provisions and drastically decreasing brood production [10]. Although exceptional protein resources such as soybean bran in *Geotrigona mombuca* (Smith) (sub *G. inusitata* in [11]) and flesh of dead animals in many *Trigona*, *Partamona*, *Oxytrigona*, *Cephalotrigona*, and others [12] can be facultatively gathered, pollen is the main protein source in bees. Obligate necrophagy (dead animal flesh is the only protein source) occurs only in *Trigona crassipes* (Fabricius), *T. hypogea* Silvestri, and *T. necrophaga* Camargo & Roubik [11, 13–15]. For *Apis mellifera*, the higher the crude protein percentage the lesser amount of pollen required to sustain production [16]. Ample protein content promotes a high birth rate and long-lived bees, 20% being the minimum protein

amount required by colonies for optimum production [17]. A well-nourished bee colony is a guarantee for good level of honey flow and breeding [16].

It is important to know pollen protein intake of meliponine bees for appropriate colony rearing (meliponiculture), as it would be useful to determine supplemental feeding needed in periods of flowering shortage. Stingless bees are important for pollination of wild and crop plants [18] and for bee-products production (honey, pollen, resin, and cerumen) [19]. The aims of the present study were to determine protein content of pollen stored in nests of three meliponine bee species (*Tetragonisca fiebrigi* (Schwarz), *Melipona orbignyi* (Guérin), and *Geotrigona argentina* Camargo & Moure) from the South American Chaco dry forest. These are three out of the seven species recorded for this area [20, 21] being important mainly for rural population for their honey, pollen masses, larvae, and cerumen [22].

2. Materials and Methods

2.1. Sampling and Study Area. Pollen mass from cerumen pots was obtained from nests of *Tetragonisca fiebrigi* (Schwarz), *Melipona orbignyi* (Guérin), and *Geotrigona argentina* Camargo & Moure from xeric forests in the Chaco region of Northern Argentina (Figure 1). Samples for protein analysis were taken from nest 7 of *T. fiebrigi*, nest 11 of *M. orbignyi*, and nests 2 and 4 of *G. argentina* from El Sauzalito (24°24'S, 61°40'W), from nest 1 of *G. argentina* from El Espinillo (25° 24'S, 60° 27'W), from nest 5 of *T. fiebrigi* from Miraflores (25°29'S, 61°01'W), and from nest 12 of *T. fiebrigi* from Villa Río Bermejito (25°37'S, 60°15'W). This Chaco dry forest is characterized by the dominance of “palo santo” (*Bulnesia sarmientoi* Lorentz ex Griseb., Zygophyllaceae) and “quebracho blanco” trees (*Aspidosperma quebracho-blanco* Schltdl., Apocynaceae), or by “quebracho colorado santiagueño” (*Schinopsis lorentzii* Engl., Anacardiaceae), “quebracho colorado chaqueño” (*Schinopsis balansae* Engl.), and “quebracho blanco” [23, 24]. Other woody elements well represented in this xerophilous flora are “mistol” (*Ziziphus mistol* Griseb., Rhamnaceae), “molle” or “guaraniná” (*Sideroxylon obtusifolium* (Roem. & Schult.) T. D. Penn., Sapotaceae), several species of *Prosopis* (“algarrobo blanco,” “algarrobo negro,” “vinal,” “vinalillo,” “carandá,” and “guachín”) (Fabaceae, Mimosoideae), “guayacán” (*Caesalpinia paraguariensis* (D. Parodi) Burkart, Fabaceae, Caesalpinioideae), “tipa colorada” or “palo coca” (*Pterogyne nitens* Tul., Fabaceae, Caesalpinioideae), “palo cruz” (*Tabebuia nodosa* (Griseb.) Griseb., Bignoniaceae), “quebrachillo” (*Aspidosperma triternatum* Rojas Acosta, Apocynaceae), “palo borracho” or “yuchán” (*Ceiba chodatii* (Hassl.) Ravenna, Bombacaceae), “palma de monte” (*Trithrinax schizophylla* Drude, Arecaceae), “meloncillo” (*Castela coccinea* Griseb., Simaroubaceae), “palo tinta” (*Achatocarpus praecox* Griseb., Achatocarpaceae), “tala” (*Celtis* spp., Celtidaceae), “duraznillo” (*Salta triflora* (Griseb.) Adr. Sánchez, Polygonaceae), “pata” (*Ximenia americana* L., Olacaceae), “molle” (*Schinus fasciculatus* (Griseb.) I. M. Johnst. var. *arenicola* (Hauman) F. A. Barkley, Anacardiaceae), “chañar”

(*Geoffroea decorticans* (Gillies ex Hook. & Arn.) Burkart, Fabaceae, Papilionoideae), “sal de indio” or “sal de monte” (*Maytenus vitis-idaea* Griseb., Celastraceae), “paloma yuyo” (*Moya spinosa* Griseb., Celastraceae), “teatín” (*Mimosa detinens* Benth., Fabaceae, Mimosoideae), “cardón” (*Stetsonia coryne* (Salm-Dyck) Britton & Rose, Cactaceae), “ucle” (*Cereus forbesii* Otto ex C. F. Först., Cactaceae), two species of *Bougainvillaea* (“rama overa”) (Nyctaginaceae), several species of *Acacia* (“tusca,” “garabato,” and “churqui”) (Fabaceae, Mimosoideae), *Capparis* (“atamisqui,” “sacha membrillo,” “sacha sandia,” “sacha poroto,” and “bola verde”), and “cardo” or “chaguar” (several Bromeliaceae genera), among others.

2.2. Pollen Analysis of Pollen Masses and Plant and Bee References. Pollen masses were dissolved in distilled water at 80–90°C and stirred with a magnetic stirrer for 10–15 min. A representative mixture of 5–10 mL was obtained and centrifuged at 472 ×g for 5 min. Processing included acetolysis [25]. Under a Nikon Eclipse E200 light microscope, a total of 300–500 pollen grains per slide were counted. Pollen grain identification was carried out comparing nest pollen slides with those present in the reference pollen collection. The reference slides consist of a total of 190 plant species and are deposited in PAL-CICYTTP pollen collection of Diamante, Entre Ríos, Argentina. It was made from flower buds of plant species collected in various localities from Chaco Province of Argentina. These plant specimens were pressed, dried, and identified by the author and deposited in the Herbarium of the Museo de La Plata (LP), the Herbarium of Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” (BA), Buenos Aires, and the Herbarium Lorentz (DTE) of Diamante, Entre Ríos, Argentina. Plant nomenclature follows [26]. Bee specimens were collected from nests, identified by Arturo Roig-Alsina, and deposited in the Entomology Collection of the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Buenos Aires, Argentina.

2.3. Determination of Protein Content. To quantify protein of pollen resources foraged by bees, certain pollen masses were selected according to their pollen type composition. Six pollen mass samples were taken from nests 5, 7, and 12 of *Tetragonisca fiebrigi*, one from nest 11 of *Melipona orbignyi*, and eight from nests 1, 2, and 4 of *Geotrigona argentina* (Table 1). For nitrogen content determination, 50 mg of pollen [27] was analyzed by the micro-Kjeldahl method [28] and crude protein was estimated using the factor 6.25 [29]. Pollen nitrogen content was analyzed in LANAI N15 (National Laboratory of Research and Services UNS-CONICET), Departamento de Agronomía, Universidad Nacional del Sur, Bahía Blanca, Argentina.

3. Results

3.1. Protein Content of Pollen Stored in Meliponine Nests. Nitrogen values from pollen grains stored in pollen pots of the three meliponine species studied varied from 1.56 to 4.86%, which is equivalent to 9.78 to 30.41% of crude

TABLE I: Main pollen types (>10% representation) present in pollen mass samples of *Tetragonisca fiebrigi* (*Tf*), *Melipona orbignyi* (*Mo*), and *Geotrigona argentina* (*Ga*) and their nitrogen and protein percentages.

Bee species	Sample	Nest and pollen mass	Main pollen types	Nitrogen (%)	Crude protein (%)
<i>Tf</i>	1	5 B	Type <i>Schinopsis</i> (59%) + <i>Trithrinax schizophylla</i> (40.5%)	3.055	19.09
	2	5 C	<i>Trithrinax schizophylla</i> (41%) + <i>Parthenium hysterophorus</i> (30%) + type <i>Schinopsis</i> (28%)	2.854	17.84
	3	5 E	<i>Trithrinax schizophylla</i> (98%)	2.898	18.11
	4	7 A	<i>Sideroxylon obtusifolium</i> (24%) + <i>Prosopis</i> (18%) + type <i>Maytenus vitis-idaea</i> (16%) + <i>Tabebuia</i> (13%)	3.126	19.54
	5	7 F	<i>Capparis speciosa</i> (42%) + <i>Mascagnia brevifolia</i> (34%) + <i>Heliantheae</i> (10%)	3.648	22.80
	6	12 C	<i>Ziziphus mistol</i> (60%) + <i>Prosopis</i> (30%)	3.542	22.14
<i>Mo</i>	7	11	Type <i>Acacia praecox</i> (53%) + <i>Prosopis</i> (38%)	3.881	24.26
<i>Ga</i>	8	4 58	Type <i>Capparis tweediana</i> - <i>C. speciosa</i> (100%)	1.56	9.78
	9	1 24	<i>Castela coccinea</i> (100%)	3.26	20.36
	10	2 31	<i>Prosopis</i> (100%)	3.34	20.87
	11	1 11	<i>Prosopis</i> (91%)	4.86	30.41
	12	2 36	<i>Prosopis</i> (89.5%)	4.32	27.03
	13	2 33	Type <i>Maytenus vitis-idaea</i> (100%)	4.23	26.87
	14	2 42	Type <i>Maytenus vitis-idaea</i> (96%)	4.24	26.51
	15	1 16	Type <i>Croton</i> (30%) + type <i>Sagittaria</i> (18%) + <i>Eleocharis</i> (12%) + <i>Castela coccinea</i> (11%)	2.65	16.59

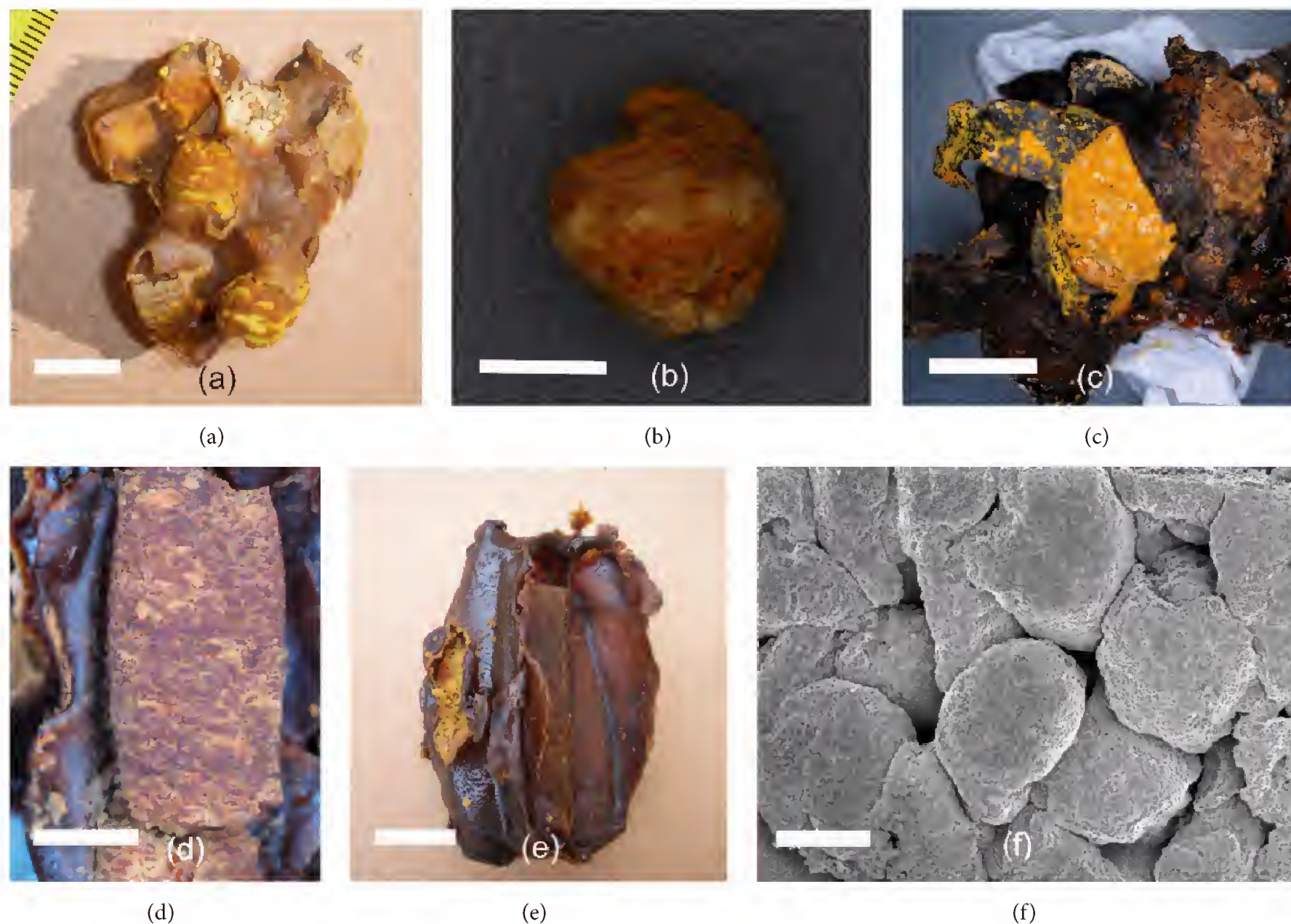


FIGURE 1: General aspect of pollen masses of *Tetragonisca fiebrigi* (a, b), *Melipona orbignyi* (c), and *Geotrigona argentina* (d, e). Individual loads from pollen masses can be seen in scanning microscope (f). Bars: (a) 10 mm; (b) 5 mm; (c) 20 mm; (d) 10 mm; (e) 20 mm; (f) 1 mm.

protein (Table 1). Higher protein values ($>20\%$) were found in samples having the following composition: *Prosopis* (samples 10, 11, and 12 of *G. argentina*), type *Maytenus vitis-idaea* (samples 13 and 14 of *G. argentina*), type *Acacia praecox* + *Prosopis* (sample 7 of *M. orbignyi*), *Capparis speciosa* + *Mascagnia brevifolia* + *Heliantheae* (sample 5 of *T. fiebrigi*), *Ziziphus mistol* + *Prosopis* (sample 6 of *T. fiebrigi*), and *Castela coccinea* (sample 9 of *G. argentina*) (Table 1). The remaining samples showed less than 20% of crude protein; the lowest value ($<10\%$) found was for type *Capparis tweediana*-*C. speciosa* (sample 8 of *G. argentina*).

Protein values found in samples composed mainly of herbs, climbers, or shrubs differed greatly among them (9.78–22.8%) and were slightly lower than those dominated by trees (17.84–30.41%). A broader range of protein values was found for *G. argentina* (9.78–30.41%) than for *T. fiebrigi* (17.84–22.80%).

4. Discussion

If protein content of pollen grains was constant for all individuals of each plant species, samples composed only or mostly of one pollen type (96–100%) (6 samples in the present study) would indicate their real protein value and it would be useful for comparing samples composed of many pollen types.

Nutritive value of pollen grains is affected by air temperature, soil moisture, pH, and soil fertility, among other factors [30] but a range of values is expected for a particular species under the same conditions [16]. The two samples dominated by type *Maytenus vitis-idaea* had similarly high protein values (26.51 and 26.87%), followed by *Castela coccinea* that ranked near 20%, the minimum limit value considered as optimal to brood development in *Apis mellifera* L. [17], and the palm tree *Trithrinax schizophylla* with slightly less than 20%. The lowest protein value was from type *Capparis tweediana*-*C. speciosa* with less than half the optimal value. Inconsistency among pollen type composition and protein value was found for the three samples dominated by *Prosopis* as pure *Prosopis* pollen showed lower protein values than those composed of 91 and 89.5% *Prosopis* and approximately 10% of other types (9.2% *Ziziphus mistol* in sample 11 and 9.5% *Capparis salicifolia* + 1% *Ruprechtia triflora* in sample 12). It appears that these accompanying pollen types were providers of important amounts of proteins. Nevertheless, the differences of protein content detected in these three samples could be due to the fact that different species of *Prosopis* (similar at light microscope) were present in their composition, as many *Prosopis* species are highly abundant in arboreal and shrubby strata of the Chaco forest and meliponine bees forage on all of them. On the other hand, soil fertility can influence nitrogen

and consequent protein composition of plant individuals [30]. The dry Chaco is a xerophytic forest that alternates with water bodies such as ancient rivers and their related riparian vegetation [31]. Nests of meliponine species here studied were sampled from these environments, and bees could forage on flowers from both dry forest patches and water bodies composed of plants growing under different nitrogen level. Moreover, livestock grazing in the forest is a very common practice in the area studied and it is responsible for increasing soil nitrogen levels through excreta. Local differences of nitrogen levels in soil might be the cause for differences in protein amounts found in pollen masses dominated by a same pollen type (the case of *Prosopis*). Fertilizer incidence was discarded, as agricultural crops were absent in the sampled area.

Stored pollen in *Apis mellifera* colonies (pollen bread) is probably fermented by lactic acid bacteria of the genera *Lactobacillus* and *Bifidobacterium* from the honey stomach added to the pollen via regurgitated nectar [6, 7]. Over and above their significance in pollen bread production and storage, these lactic acid bacteria are important against pathogens and production and storage of honey [6, 9]. These microbial symbionts are also present in its ecologically similar and closely related group: the stingless bees (Meliponini) [9, 32]. Proteins, mainly enzymes, are secreted by this beneficial microbiota during stress [33], which could increase the protein content of stored pollen. Versatile digestive physiologies characterize broad polylectic bees [34], as pollen of diverse protein spectrum seems to be similarly foraged by these highly social species. Similar to other studies on bee foraged pollen [35, 36], protein content of pollen types was variable. It is likely that amino acids deficiencies of certain pollen are compensated by randomly foraging on a broad spectrum of pollen plants. This is in agreement with the argument stated by [37] that nutritive value of pollen for bees is not directly correlated with protein quantity since a qualitative factor is of greater importance. For instance, [38] detected low protein levels in hand-collected pollen from desert plants (from 7 to 15.6%), but they highlighted that their amino acid patterns were in agreement with the requirements for honey bees. An interesting topic to be tested by experimental studies is on the capability of meliponine brood to develop by eating pollen having the half of the optimal protein level for *Apis mellifera*. However, palynological surveys on meliponine bees from the Chaco region showed that scarce number of pollen masses are composed of pure or nearly pure pollen from *Capparis tweediana* or *C. speciosa*, having only 9.78% of protein (only six out of 75 masses analyzed of *G. argentina*, but none in 86 masses of *T. fiebrigi* or in ten of *M. orbignyi*) [39, FGV unpublished data]. Pollen with low protein levels would expose bees to more severe amino acids deficiencies. However, bees can be well developed when feeding on high amounts of these pollens, but a colony would be threatened when there is a low amount of pollen stores or shortage of flowerings [16]. Meliponine bees store great amounts of pollen (353 g in 20 pollen pots of diverse filling in one nest of *G. argentina*) [39] (Figure 1).

An attempt was made to determine the differences in protein content due to life-form of plants foraged and season

when sampling was carried out and among bee species but further studies are necessary. However, most protein-rich pollen species were woody, in accordance with findings for other semiarid areas of Argentina [35] but findings by [36] did not show this pattern. It is widely known that *Solanum* species are protein-rich pollen resources, ranging from 34.1% for *S. tabanoense* Correll to 54.9% for *S. lycopersicum* L. [40]. *Solanum* was highly foraged by *Melipona orbignyi* [FGV unpublished data]. The only sample studied for *M. orbignyi* showed a protein value greater than the one required for *A. mellifera* and was dominated by types *Acacia praecox* and *Prosopis* followed by four types of less than 4% representation. As this species also prefers *Solanum* and other protein-rich pollen from poricidal anthers whose grains are easily gathered by vibratile buzzing, more samples would need to be analyzed to establish whether protein requirements are high for this *Melipona* species. The genus *Solanum* is well represented in the dry Chaco (more than 10 species), mainly in open areas of nitrogen-rich soils and water bodies.

It is surprising that pollen showing the highest protein content (>26%) belonged to highly nectariferous plants well represented in meliponine and *Apis* honey in this region such as *Prosopis*, *Maytenus*, and *Ziziphus* [39, 41–45]. Studies on protein content of pollen loads and pollen analysis of honey carried out in other semiarid areas also show this tendency, as seen in Table III of [35] and Table II of [46] for *Condalia*, Brassicaceae and *Prosopis*, in Table III of [36], and in Table II of [47] for *Adesmia*, Rosaceae, *Trifolium*, *Melilotus*, *Schinus*, and Brassicaceae. Furthermore, pollen from the nectariferous *Larrea* and *Prosopis* showed the richest protein value in a study carried out in a North American desert [38]. This pattern of high protein value in pollen of highly nectariferous plants foraged by honey-producing bees is here hypothesized and should be further studied.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Observations on the Biology and Anatomy of Myerslopiidae (Hemiptera, Membracoidea)

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Adults and nymphs of *Mapucheia chilensis* (Nielson), from the poorly known family Myerslopiidae, were collected from the litter horizon of temperate forests and shrub bogs in southern Chile. The species apparently feeds on roots and creeping stems of angiosperms. Salivary sheaths of captive specimens terminated in vascular bundles. Indirect evidence suggests feeding on phloem sap. Both nymphs and adults are strong jumpers and both actively disperse, as evidenced by their capture in pan traps. The Malpighian tubules of this species produce no brochosomes and, unlike in most previously studied Membracoidea, comprise no specialized secretory segment. Each tubule comprises secretory cells scattered among excretory ones, a condition not previously known among Hemiptera.

1. Introduction

The family Myerslopiidae includes three genera, two in New Zealand and one in Chile, altogether containing 19 morphologically similar species [1]. Adult myerslopiids are stout, strongly sclerotized insects, 3 to 7.5 mm in length, with elytra-like, medially sealed tegmina, and without traces of hind wings underneath. Both adults and nymphs dwell cryptically on or near the ground, and their integument, sculptured with ridges and protuberances, is usually camouflaged with hardened soil (Figures 1(a)–1(h)). Members of the family have formerly been classified as a tribe within the leafhopper subfamily Ulopinae (Cicadellidae) but more recently were recognized as a sister lineage to the rest of Membracoidea (leafhoppers + treehoppers), which shares some plesiomorphic traits with cicadoids and cercopoids [2]. The disjunct range of the group has been interpreted as evidence of its considerable age and association with Gondwanaland [1, 2].

Until now the main sources of information on the myerslopiids biology were collecting records of the New Zealand species, the majority of which described the habitat as simply “moss” or “litter” [3]. This led Hamilton to assume that myerslopiids, like peloridiids, are “associated with moss rather than with higher plants.” Bryophagy has not been known in Cicadomorpha and is exceptionally rare among other

Auchenorrhyncha [4]. Other authors referred to myerslopiids as possible fungivores [1] or even predators [5]. The life history of myerslopiids thus merits clarification.

Also of interest is the activity of the Malpighian tubules of myerslopiids. Secretions of these organs play important roles in the biology of different groups of Cicadomorpha [6–8]. For example, cicadellids actively coat their integuments with water-repellent sheaths of brochosomes, proteinaceous products of specialized Malpighian tubule segments [9–12]. It has been argued that the production of brochosomes had evolved in the common ancestor of Membracoidea [13]. Because myerslopiids have been identified as being among the few leafhopper taxa lacking brochosomes [14], data on their Malpighian tubules are much needed.

An expedition to temperate Chile during the austral summer of 2013–2014 gave the author an opportunity to clarify the microhabitat of myerslopiids, observe behaviors of captive specimens, and preserve material for anatomical studies. A note on the myerslopiids karyotype and tests has already been published [15]. Here, I present ecological, behavioral, and anatomical observations to demonstrate that myerslopiids present no significant departures from cicadellids in their life history traits, but their Malpighian tubules have a different organization, not previously known among Hemiptera.

2. Methods

Collecting was done mostly by manual sorting of detritus on a white plastic sheet. At the Senda Darwin biological station, additional specimens were collected in water-filled white or yellow disposable plastic dishes placed on the ground as pan traps. Most of the collected specimens were kept live in ventilated plastic food containers on plant cuttings, moss, rotten wood, and detritus from the source microhabitat. Their behaviors were intermittently observed under a stereomicroscope.

Adults and nymphs collected at Senda Darwin were caged on cuttings of *Griselinia racemosa* roots, identified as a likely food source. To examine salivary sheaths, roots segments where the insects had been observed feeding were marked with a pen marker and then excised, fixed in 4% formaldehyde for 24 hours, and stored in 70% ethanol. These root chunks were subsequently embedded into paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin.

The ultrastructure of the Malpighian tubules was examined in two adult females, one adult male, and two 3rd-instar nymphs. The specimens were preserved in 2.5% glutaraldehyde in PBS buffer, pH 7.4. The tubules were dissected out, stained with osmium tetroxide, embedded in Epon, and sectioned into 60 nm sections. The sections were poststained with uranyl acetate and lead citrate and examined on a JEM-1011 transmission electron microscope (JEOL Ltd., Japan).

3. Results

3.1. Collected Material. Chile: *Huerquehue National Park*, N of Lake Tinquilco, head of the Lagos trail, 39.155°S, 71.716°W, mature *Nothofagus* forest with *Chusquea* understory (Figure 2(a)), 20–25 Dec., 2013, 10 adults and 5 nymphs from leaf litter with *Hydrangea serratifolia*, *Dendroligotrichum dendroides*, and small ferns (Figure 2(b)); same site, 9–10 Feb., 2014, 11 adults and 1 nymph (2nd instar). *Puyehue National Park*, sector Anticura, near park entrance, 200 m N of Road 215, 40.667°S, 72.174°W, 15–17 Jan., 2014, >25 adults and 5 nymphs (5th instar) from ca. 3 m² of leaf litter under a tree, with abundant *Hydrangea serratifolia* and some herbaceous plants (Figures 2(c)–2(e)); trail Princesa, 40.665°S, 72.172°W, 15 Jan., 2014, 3 nymphs (5th instar) from forest leaf litter along trail; trail Chile, 40.669°S, 72.170°W, 18 Jan., 2014, 2 adults and 1 nymph (5th instar) from forest leaf litter with *Blechnum*, *Hydrangea*, and *Dendroligotrichum*; trail Salto del Indio, mature *Nothofagus* forest, 19 Jan., 2014, 4 adults from leaf litter. *Alerce Andino National Park*, sector Sargazo, near park entrance, 41.508°S, 72.621°W, open slope near forest edge, 6 Jan., 2014, 1 nymph (5th instar) from short grass and moss under *Drimys*; the same site, *Drimys* forest with *Chusquea*, 8 Jan., 2014, 2 adults from litter with *Dendroligotrichum* and creeping vines. *Chiloé, Senda Darwin biological station*, forest, postfire bog with abundant shrubs (*Baccharis*, *Tepualia*, and *Gaultheria*), large ferns (*Blechnum* spp., *Gleichenia squamulosa*), and trees (Figures 2(f) and 2(g)), 41.88°S, 73.67°W, 11 Jan., 2014, 3 nymphs (2nd and 3rd instars) from inside the spongy mass of decomposing old fronds around “trunks” of large *Blechnum magellanicum*;

the same site, 27 Dec., 2013–4 Jan., 2014, 6 nymphs (5th instar) from accumulation of litter and peat under *Podocarpus* and large ferns, with abundant *Griselinia racemosa* roots; the same site, 13 Jan., 2014, and 1–7 Feb., 2014, 5 adults and 6 nymphs (4th and 5th instars), captured in plastic pan traps (Figure 2(g)), mostly on rainy nights. *Chiloé National Park*, sector Cucao, shrub bog along the Lahuan trail (Figure 2(h)), 42.621°S, 74.107°W, 13 Jan., 2014, 1 nymph (5th instar) from peat with roots; the same site, 3 Feb., 2014, 2 adults from litter and detritus under large ferns and trees; sector Rancho Grande, 42.572°S, 74.075°W, 3 Feb., 2014, 1 nymph from moss, coll. V. Hartung.

The specimens varied in size, sculpturing, and coloration between and within the collecting sites but were all assigned to *Mapucheia chilensis* (Nielson). The type locality of that first South American myerslopiid to be described is in the Anticura sector of the Puyehue NP [5]. Szwedo [16] made it the type of his genus *Mapucheia*, in which he described two additional species, each based on a single specimen: *M. burckhardti*, from Bosque de Fray Jorge NP, which is the northernmost outpost of Valdivian temperate rain forests, and *M. hamiltoni* from the Huerquehue NP. The specimens I collected in the Huerquehue NP did not consistently differ from the specimens collected elsewhere.

3.2. Observations of Caged Insects. Due to their cryptic habitat and camouflage, the insects could not be observed in nature. Caged adults and nymphs survived indefinitely until killed. They walked slowly but most of the time clung motionlessly to plant stems or roots, occasionally expelling droplets of clear excrement, which was interpreted as a sign of feeding. The insects were not observed feeding or staying long on green plant parts but twice were observed feeding on damp rotten wood. When disturbed, both nymphs and adults jumped strongly. In one adult, a jumping distance of 50 cm was recorded. The jumps ended by crash-landing followed by brief immobility. Two captive 5th instar nymphs molted to the adult stage. One of these (Figure 1(c)) was closely watched for 9 hours following the molt, during which it remained inactive. No attempts to coat the integument with soil or plant debris were noticed in either of molted specimens during the days following the molt. Grooming, courting, and mating were not observed.

3.3. Salivary Sheaths. Seven salivary sheaths were tracked, all of them terminating in the vascular bundle of the rootlet. Among these, two penetrated into the xylem (Figures 3(a) and 3(b)), while the rest were difficult to interpret, terminating equivocally between the phloem and the xylem (Figures 3(c) and 3(d)) or being poorly preserved. One branched sheath was observed (Figure 3(a)).

3.4. Malpighian Tubules. The general arrangement of the digestive tract and four Malpighian tubules is as typical for Cicadomorpha (Figure 4). The shape of the Malpighian tubules is identical in adults and nymphs. Each tubule gradually widens towards the base but becomes constricted again for a short length before entering the filter chamber.

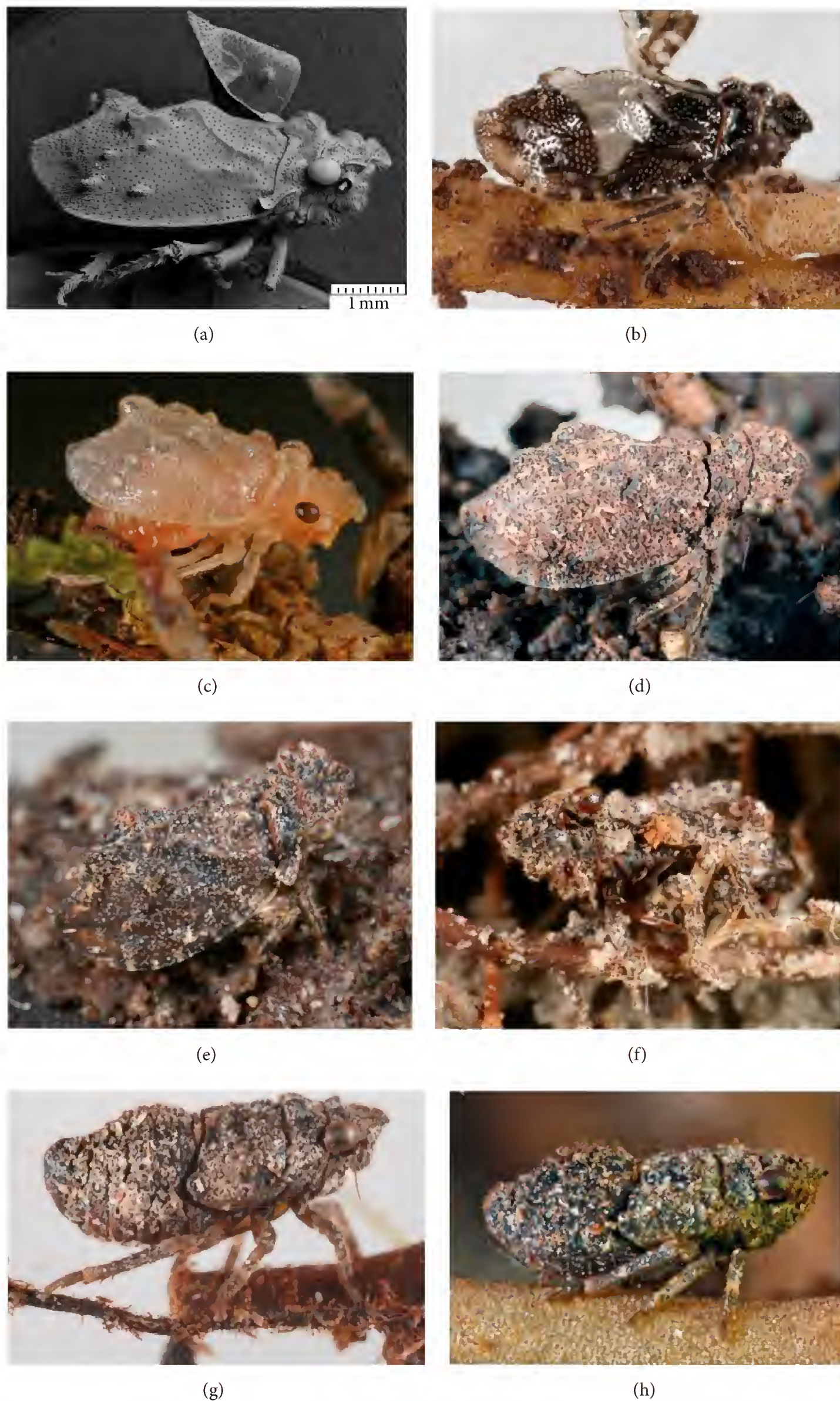


FIGURE 1: *Mapucheana chilensis*. ((a), (b), (c), and (d)) Specimens from the Huerquehue NP. (a) Scanning electron image of a female molted in captivity and not camouflaged with soil; the left tegmen is mangled. (b) The same specimen live, showing color pattern. (c) Freshly molted female. (d) Camouflaged specimen. (e) Specimen from the Puyehue NP. (f) Soil camouflage in combination with disruptive coloration of legs makes this specimen hard to see; note that the eyes remain clean. (g) 5th instar nymph. (h) 3rd instar nymph. All photos of live specimens were taken in captivity.



FIGURE 2: *Mapuchea chilensis* habitats (collecting sites). (a) *Nothofagus* forest N of the lake Tinquilco in Huerquehue NP. (b) The same forest floor with ferns and *Hydrangea* sp. (c) Puyehue NP near Anticura campground, leaf litter near a tree base, with abundant *Hydrangea serratifolia*. (d) The same, detailed view; note the absence of mosses and ferns. (e) The same, with litter removed to expose creeping stems of *H. serratifolia*; also note the abundant volcanic ash deposited upon the 2011 Puyehue-Cordón Caulle eruption. (f) Shrub bog at the biological station Senda Darwin on Chiloé. (g) The same, showing pan traps. (h) Shrub bog in Chiloe NP near Cucao.

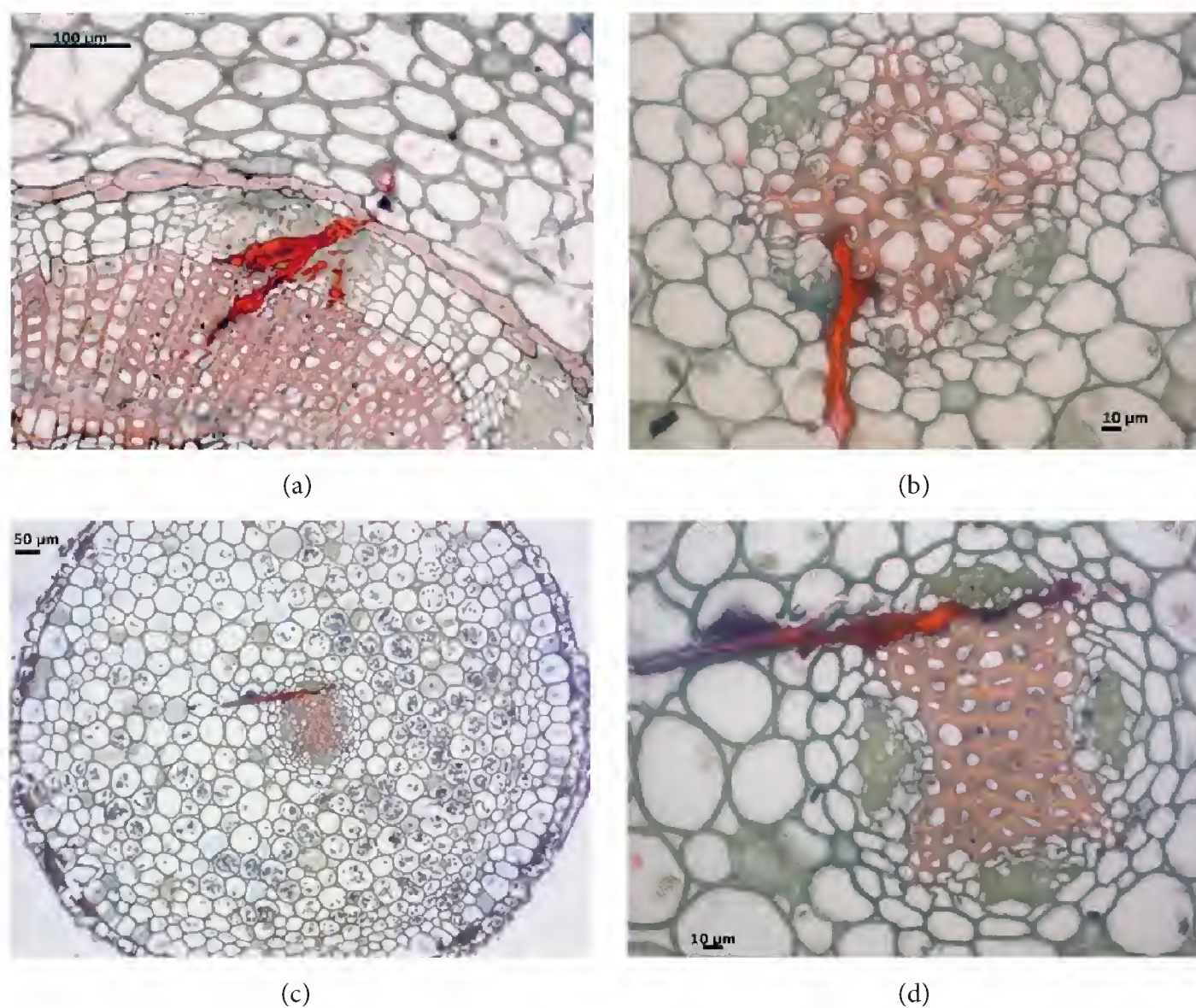


FIGURE 3: Histological sections of *Griselinia racemosa* roots with salivary sheaths of *Mapucheia chilensis*. (a) Branched sheath, with one branch penetrating into the xylem. (b) Sheath terminating in the outermost layer of the xylem. ((c) and (d)) Sheath terminating equivocally, between the phloem and the xylem.

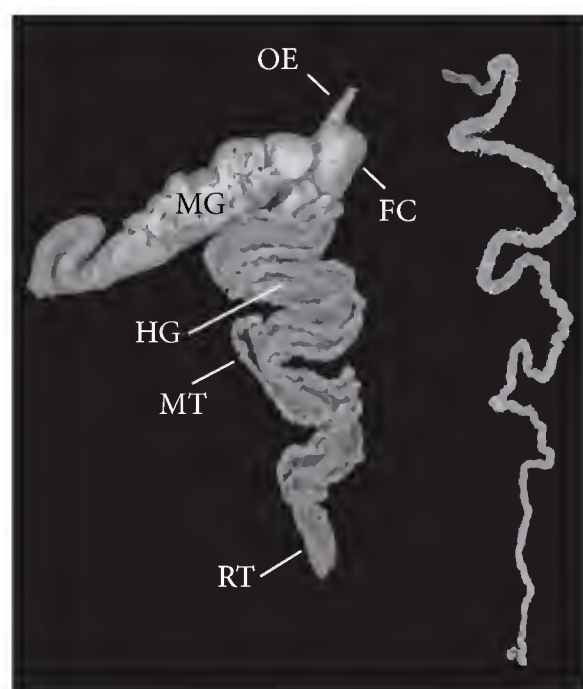


FIGURE 4: *Mapucheia chilensis* digestive tract with the Malpighian tubules and an isolated tubule (omitting the basal part entering the filter chamber). OE: oesophagus; FC: filter chamber; MG: midgut; MT: Malpighian tubules; and RT: rectum.

The exposed length of the tubule is convoluted and nodulose, resembling twisted rope, distally less so (Figure 4). The tubules form two pairs, the termini of each pair united in a loop attached to the rectum.

In both nymphs and adults, the exposed parts of the tubules comprise two types of cells, excretory and secretory. In the thicker, more basal part of the tubule, secretory cells are interspersed among more numerous excretory cells. As the tubule narrows distally, the secretory cells gradually disappear; the most distal part seemingly comprises only excretory cells.

The excretory cells have an extensively developed labyrinth of basal membrane infoldings (Figures 5(a) and 5(b)). Their apical membrane bears a brush of long, simple, or lamellar microvilli, containing mitochondria, and forms intracellular canaliculi, some of them branching or forming flask-shaped cavities (Figures 5(b) and 5(c)). These cavities are also lined with microvilli or lamellae (Figure 5(d); compare with Figure 8 in [17]). The cytoplasm contains numerous conspicuous mitochondria and, in some cells, deposits of glycogen granules (Figure 5(d); compare with [18]).

The secretory cells (Figures 6(a)–6(h)) are smaller and lack apical microvilli and conspicuous mitochondria. They contain numerous secretory vacuoles filled with spherical or somewhat irregular dense granules, ca. $0.2\ \mu\text{m}$ in diameter, with coarsely granulose surface (Figure 6(f)), and, occasionally, smaller particulate material. The $0.2\ \mu\text{m}$ granules are also present in the tubule lumen (Figure 6(c)). The granules are formed in Golgi cisternae (Figures 6(g) and 6(h)). Most of the remaining cytoplasm is filled with cisternae of endoplasmic

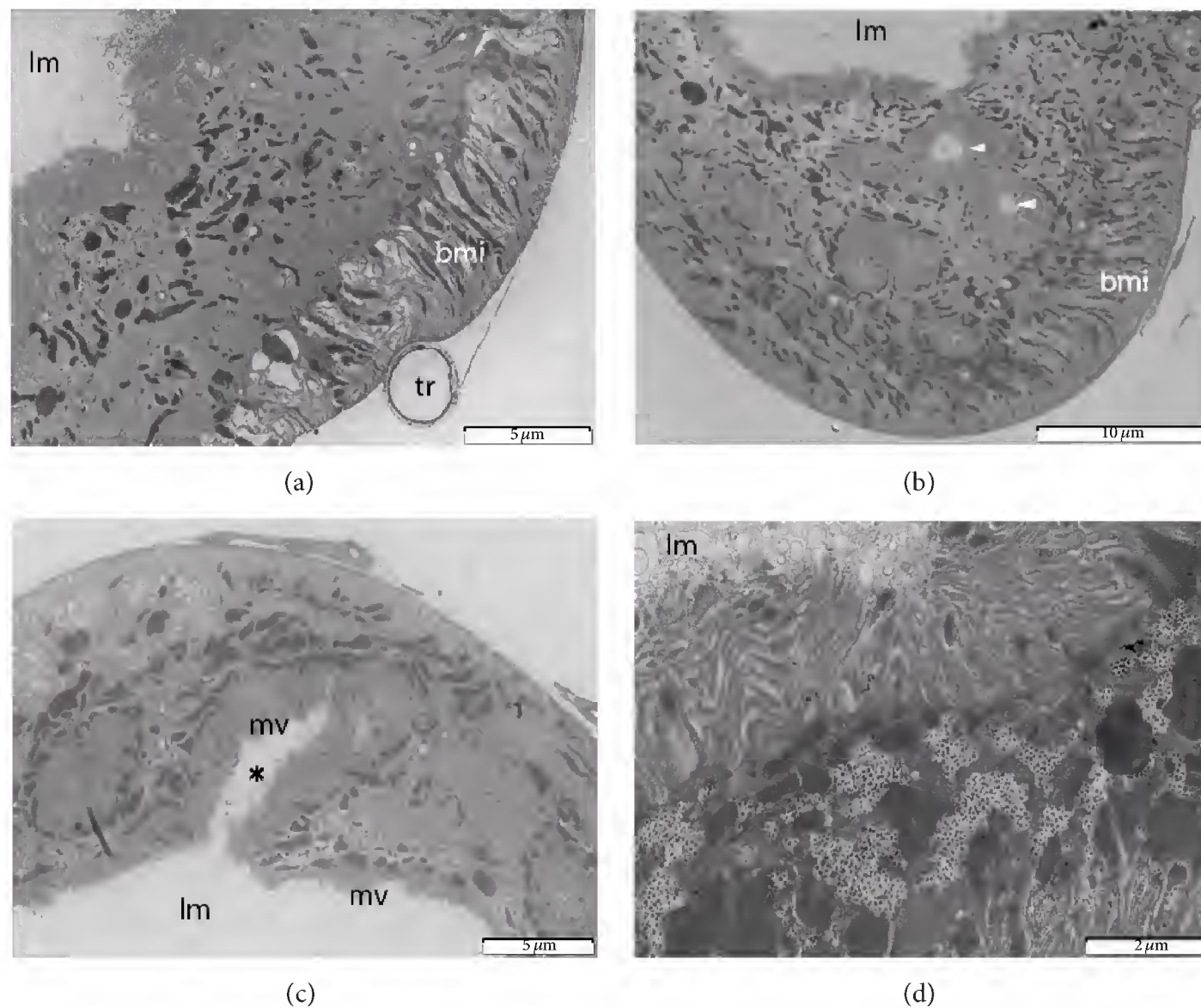


FIGURE 5: Excretory cells of the Malpighian tubules of *Mapucheia chilensis*. ((a)–(c)) Adult insects, general structure of the cells. (d) 3rd instar nymph, an excretory cell showing deposits of granular glycogen and lamellar microvilli bordering an intracellular canaliculus. lm: tubule lumen; tr: trachea; and bmi: basal membrane infoldings. Intracellular canaliculi are shown with arrowheads in (b) and an asterisk in (c).

reticulum containing homogeneous material (Figure 6(d), arrowheads).

4. Discussion

4.1. Habitats and Hosts. *M. chilensis* was collected in temperate Chile from forests and shrub bogs. This is similar to the New Zealand myerslopiids, which also occur in both forested and open habitats [3]. *M. chilensis* dwells cryptically on the ground or within decomposing plant material. On bogs, where the litter horizon is not well defined, the insect can be found at considerable depths within the peat. Most of the microhabitats where *M. chilensis* was found contained roots or stems of angiosperms, which apparently served as the food source. At several forest locations in the Huerquehue, Puyehue, and Alerce Andino parks, the insects were found between creeping stems of the vine *Hydrangea serratifolia*. It grew abundantly at the site in Anticura where the largest numbers of *M. chilensis*, 30 specimens, were collected (Figures 2(c)–2(e)). In at least two sites on the bog at Senda Darwin (Figure 2(f)), *M. chilensis* appeared to be associated with surface roots of the shrub *Griselinia racemosa*, abundantly ramifying within the litter and peat. Interestingly, like myerslopiids, the genus *Griselinia* has a disjunct distribution between southern South America and New Zealand. Caged insects were observed feeding on both *G. racemosa* roots

(Figures 3(a)–3(d)) and *H. serratifolia* stems. No single plant species was present at every collecting site, indicating that *M. chilensis* is not associated with one host, but all such sites contained angiosperms. Most of the previous records did not associate myerslopiids with angiosperms, although three New Zealand species have been collected, among other places, from *Celmisia* and mats of *Raoulia*, both of Asteraceae [3]. Ferns were present at most of the collecting sites (Figures 2(b) and 2(g)) but were absent from the Anticura site (Figures 2(c)–2(e)). Curiously, three nymphs were found within dead plant material coating the “trunks” of large ferns that microhabitat could potentially contain epiphytes. A single record of a New Zealand myerslopiid from a fern has been listed [3].

Previous records consistently associated myerslopiids with mosses [3]; yet mosses were completely absent from the *Mapucheia*-rich site in Anticura (Figures 2(c)–2(e)). The mosses on the bogs of Chiloé (Figures 2(f)–2(h)) were intensively sampled, yielding abundant peloridiids ([19]; V. Hartung, personal communication), but only occasional individuals of *M. chilensis*. The latter species was consistently found on those same bogs in moss-free detritus accumulated under shrubs, large ferns, and trees.

4.2. Feeding Mode. Salivary sheaths revealed that the insects fed by tapping into the vascular tissue. This feeding mode is consistent with the presence of a filter chamber and general

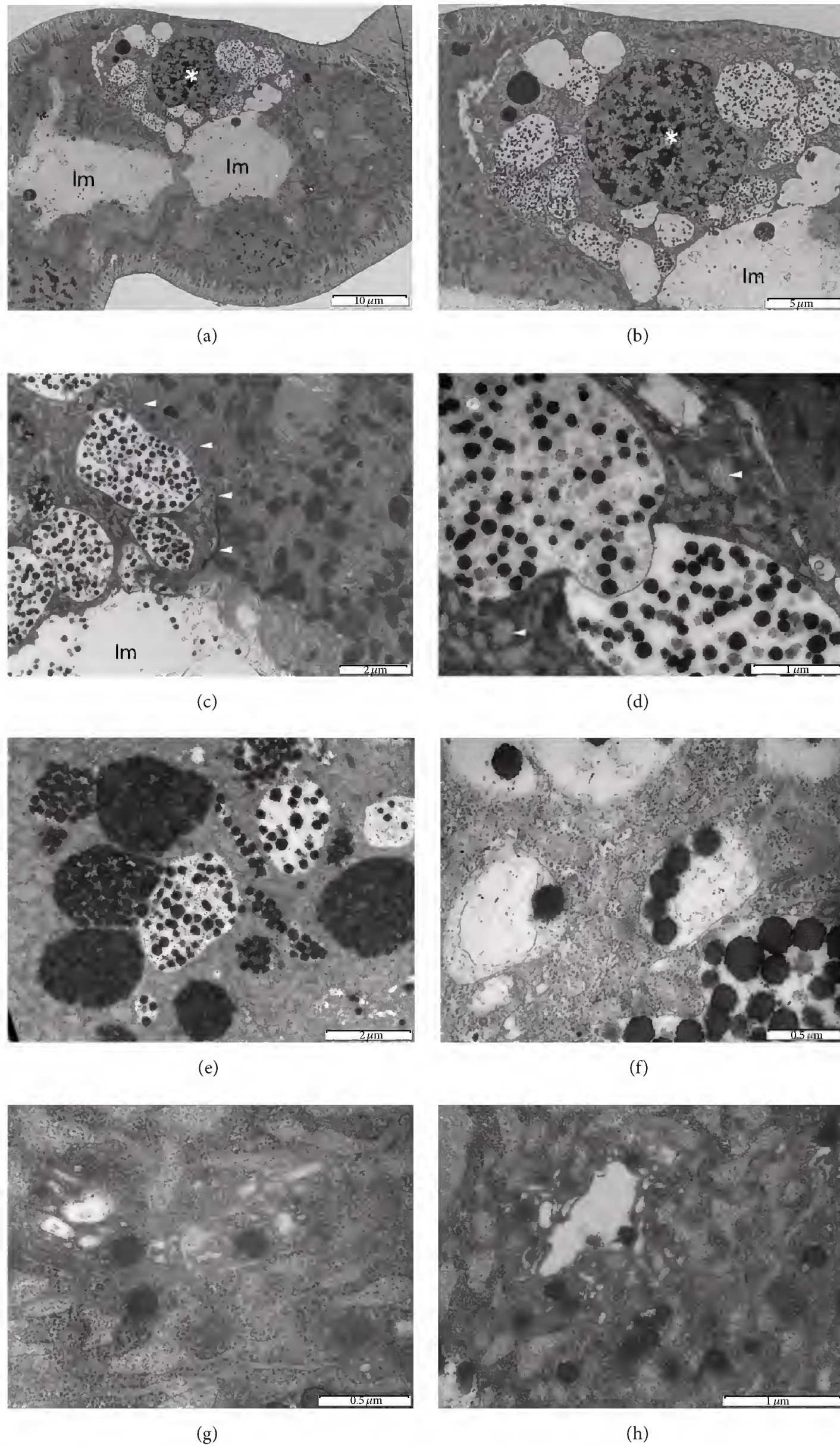


FIGURE 6: Secretory cells in the Malpighian tubules of *Mapucheia chilensis*. ((a)–(d)) Cells in the tubules of a 3rd instar nymph. (a) A single secretory cell (its nucleus marked by an asterisk) among excretory cells. (b) The same cell, closeup. (c) Contact between a secretory and an excretory cell; arrowheads point at the junction. Note secretory granules in the tubule lumen. (d) Secretory vacuoles with granules and additional particulate material. Arrowheads point at cisternae of endoplasmic reticulum. ((e)–(h)) Cells in the tubules of an adult. (e), (f) Secretory vacuoles variably loaded with granules. (g), (h) Golgi regions with developing granules. lm: tubule lumen.

structure of the gut and Malpighian tubules, similar to those in previously studied Cicadomorpha (Figure 4). However, the exact source of the ingested sap, phloem or xylem, could not be identified based on the sheaths because of the small size of the sample. Salivary sheaths mark not only feeding, but also probing behavior, thus requiring many observations in order to make inferences. For example, among the 389 examined salivary sheaths of *Philaenus spumarius* (L.) examined by Horsefield, only 60% terminated in the xylem [20].

Although some of the observed salivary sheaths terminated in the xylem (Figures 3(a) and 3(b)), the external morphology of myerslopiids indicates xylem-feeding to be unlikely. The low nutritional value of xylem sap requires xylem-feeders to intensify ingestion by increasing the volume of cibarial pump muscles [21]. Externally this is manifested in the frontoclypeus being disproportionally enlarged in all stages, but especially so in young instars. The head proportions of myerslopiids follow the opposite developmental trajectory, with the frontoclypeus becoming progressively more inflated towards the adult stage (Figures 1(a) and 1(h); also mentioned in [16]). Therefore, the most likely food source is phloem sap.

4.3. Locomotion and Other Behaviors. While adults jump in all the Cicadomorpha except cicadas, nymphal jumping is thought to have evolved in the ancestral membracoidea and been subsequently lost in the lineage that includes several leafhopper subfamilies and treehoppers [13]. The evidence of jumping by both adult and immature *Mapuchea* is, therefore, not surprising. It is worth mentioning that the leafhopper subfamily Ulopinae, to which myerslopiids have been assigned in the past, contains *Ulopa* species with nonjumping nymphs.

The fact that adults and nymphs of *M. chilensis* were consistently captured in pan traps (Figure 2(g)) suggested that they emerged to the surface in order to disperse. The insects quickly drown because their integument is not water-repellent. Climbing or accidentally falling from vegetation into these traps was unlikely, suggesting that the trapped insects had been moving around by hopping.

The origin of the characteristic soil crust of myerslopiids remains a mystery. Its consistent presence on the majority of collected specimens (Figures 1(d)–1(h)) suggests that it may be actively produced, but my limited observations did not discover any such behavior. Similar crusts have been reported for the leafhopper genera *Evansiola* China and *Paulianiana* Evans, formerly allied with myerslopiines within Ulopinae [22].

4.4. Malpighian Tubules. It has long been known that the Malpighian tubules of Membracoidea are unique among Hemiptera in having, in their middle or somewhat more distally, a glandular segment [23]. It is usually inflated throughout the life cycle, but in adults of some leafhoppers it becomes secondarily deflated. This segment has been observed in all the previously examined Cicadellidae (e.g., [23, 24]), Membracidae ([23, 24], Rakitov unpublished), and

Melizoderidae (Rakitov, unpublished), but not in Aetalionidae (Rakitov, unpublished). The glandular segment consists entirely of specialized secretory cells and is flanked on either side with narrow, nodulose tubule segments consisting entirely of excretory cells [17]. In most adult cicadellids, the secretory cells produce brochosomes, proteinaceous particles of curious structure, which are released after molts and applied in special behaviors onto the integuments (reviewed in [11]). In some cicadellid subfamilies, the glandular segments secrete brochosomes starting from the 1st nymphal instar, while in others they produce other diverse products prior to switching, during the last nymphal instar, to production of brochosomes. These Malpighian tubule secretions of leafhopper nymphs include granules, tubules, or colloids [25]. Their chemistry and function are unknown, but some leafhopper nymphs apply these secretions onto their bodies [26]. Similar products have been found in the glandular regions of the immature and adult Membracidae and Melizoderidae (Rakitov, unpublished). It has been argued that treehoppers have evolved from within leafhoppers and quit secreting brochosomes secondarily [13].

Unlike in most previously studied Membracoidea, the Malpighian tubules of *M. chilensis* have no glandular segment composed of secretory cells (Figure 4). Instead, the tubules of both nymphs and adults have secretory cells interspersed among excretory cells along the length of tubule, except in the terminal region. The secretory cells produce simple granules, ca. 0.2 μm in diameter, which accumulate in large numbers in secretory vacuoles prior to being released into the lumen (Figures 6(a)–6(f)). This resembles the granular secretions produced in the Malpighian tubules of some cicadellid nymphs prior to switching to the production of brochosomes [25]. In both cases, the granules form in Golgi cisternae (Figures 6(g) and 6(h)), suggesting their proteinaceous nature. In the absence of chemical data, it is impossible to conclude whether the secretory products of *M. chilensis* are related to those of cicadellid nymphs, but this seems to be a possibility, given their structural similarity and the fact that Myerslopiidae and Cicadellidae are close relatives. The tubule excretory cells of *M. chilensis* (Figures 5(a)–5(d)) are identical to those of cicadellids [17] and represent a generic type common among insects. Therefore, the essential difference between *M. chilensis* and the previously studied Membracoidea concerns how the secretory cells are situated along the tubule: interspersed in the first case and forming a compact zone in the second.

Interspersed secretory cells, of unknown function, have been observed in the Malpighian tubules in other insect orders [27] but, to the best of my knowledge, not in Hemiptera. The closest living relatives of Membracoidea are spittlebugs (Cercopoidea) and cicadas (Cicadoidea), the three groups forming together the infraorder Cicadomorpha. During the nymphal stage, the Malpighian tubules of spittlebugs and cicadas produce secretions, spittle components in the first group [6, 7, 28–30] and the so-called anal liquid, of uncertain function, in the second [8]. In both groups, the tubules have a similar organization, representing a third type, different from that of either *M. chilensis* or other Membracoidea. Each has a

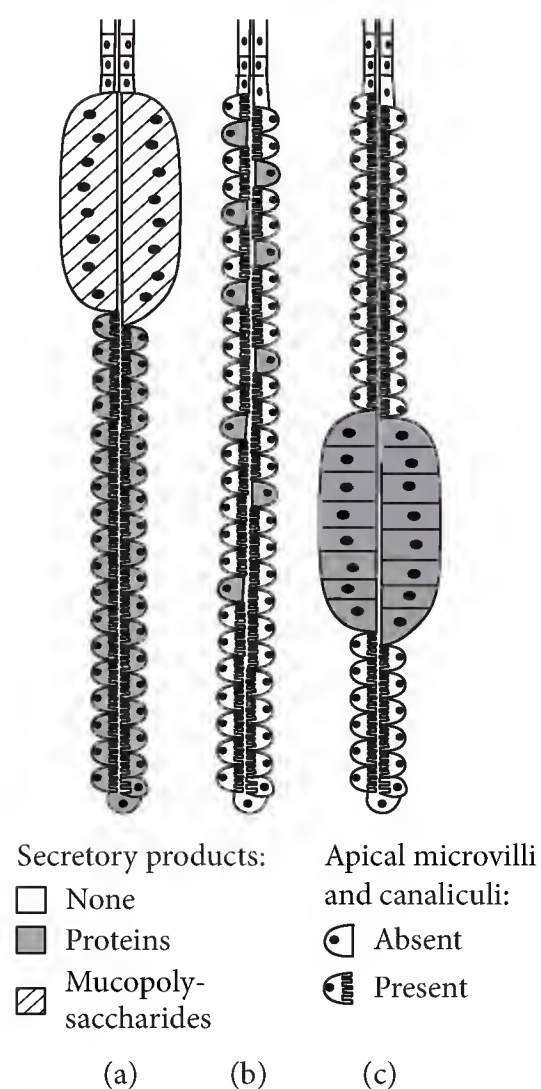


FIGURE 7: General organization of the Malpighian tubules in Cicadomorpha. The most basal parts, hidden inside the filter chamber, are not shown. (a) Immature Cercopoidea and Cicadoidea. (b) *Mapucheia chilensis*. (c) Cicadellidae and Membracidae.

short inflated glandular basal segment, composed of secretory cells producing mucosubstances, and a long nodulose distal segment, composed of essentially excretory cells, with characteristic apical microvilli and intracellular canaliculi, which additionally produce proteinaceous granules [8, 28–31].

The basal parts of the tubules show no secretory modification in Membracoidea, including *M. chilensis*, but their more distal parts secrete proteinaceous substances in all Cicadomorpha, representing three ways (Figures 7(a)–7(c)) to distribute the excretory and secretory functions along the tubule: (1) cells homogenous, each combining the secretory and excretory functions (spittlebug and cicada nymphs); (2) secretory cells interspersed among excretory cells (*M. chilensis*); and (3) excretory and secretory cells segregated into separate segments (Cicadellidae and Membracidae).

Like in *M. chilensis*, the Malpighian tubules of Aetalionidae have no morphologically distinct glandular region (Rakitov, unpublished), but their cell composition and ultrastructure have not yet been examined.

I have argued in the past that the secretory specializations of the Malpighian tubules together with the habit of applying their secretions onto the body, displayed by spittlebugs, cicadas, and membracoids, have a common evolutionary origin [8]. The fate of the tubule secretory products of *Mapucheia* is unknown; no postmolt behaviors similar to anointing of leafhoppers and treehoppers [26] was observed, but the paucity of observations makes conclusions premature.

Myerslopiidae merit further study in this regard, as they may represent the only recent group of Membracoidea which had separated prior to and thus may shed light on the origin of brochosomes and related behaviors of leafhoppers.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Dipterofauna Associated with *Sus scrofa* Linné, 1758, Carcasses in Urban and Coastal Regions of São Paulo State, Brazil

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Cadaverous entomofauna successions vary according to the region, environment, and climate, and such differences may occur within the same country due to seasonal variations. The present study aimed to analyze and compare the dipterofauna that visit or colonize carcasses in the urban and coastal areas of São Paulo, Brazil, during summer and winter seasons. Four swine (*Sus scrofa* Linné, 1758) carcasses of approximately 12 kg were used. The animals were previously euthanized and then placed in metal cages covered with a flight intercept trap (Shannon, modified). In total, 10,495 flies from 39 families were collected, with 15 species belonging to the Calliphoridae family, 14 species belonging to the Fanniidae family, 43 species belonging to the Muscidae family, and 22 species belonging to the Sarcophagidae family. Flies from these four families visited all carcasses; however, they did not show the highest visitation frequencies in all of the trials. Species variations occurred between the experiments that were performed at different locations and in different seasons. Furthermore, difference in the number of insects attracted to each stage of decomposition was observed. In addition to the four families highlighted above, the families Phoridae, Sepsidae, Otitidae, and Piophilidae were observed in all carcasses.

1. Introduction

Forensic entomology is the study of insects and arthropods in relation to legal issues, particularly death investigations [1]. When combined with a criminal investigation, the analysis of arthropods associated with corpses can add information, help solve crimes and elucidate their circumstances, and determine the postmortem interval (PMI), thus linking suspects to crime scenes, demonstrating the movement of corpses, or determining the levels of drugs consumed by the deceased [2]. Adult or immature insect specimens should be considered physical evidence that is as important as the victim's own biological material [3]; therefore, it is necessary to handle these specimens with the appropriate techniques

to prevent the destruction and contamination of insects and preserve the collection [1].

The majority of invertebrate fauna associated with carcasses consist of dipteran and coleopteran insects that are attracted by different decomposition stages occurring in the body, and their presence generates a succession of complex communities composed of scavenger species and their predators and parasites [4]. The duration of each phase or stage of putrefaction during cadaver decomposition can differ depending on the location and conditions of the carcass/corpse; however, the order of the events remains constant [5]. In tropical countries, the high typical temperatures shorten the time between death and skeletonization relative to what occurs in temperate regions and result in a shorter

period for data and material collection [6]. In addition to variations in decomposition duration, the variety of species involved in the decomposition process can vary according to climate [4], region [7], altitude [8], and vegetation [9], and variations may occur in the same location during different seasons [10, 11].

In South America, the literature on cadaverous entomofauna is relatively limited [12], especially in Brazil, which has limited available published entomological data related to carcasses in different regions of the country. Papers have been published on studies in Curitiba [13], Campinas [14], and Rio de Janeiro [15], among others.

To increase the knowledge of insect fauna that visit and/or colonize carcasses, two locations that have been previously unexplored by forensic entomological studies were chosen. The present study aimed to compare the cadaverous entomofauna in the city of São Paulo and the town of Peruíbe over two seasons (summer and winter). This research focused on the Diptera order and the Calliphoridae, Muscidae, Fanniidae, and Sarcophagidae families because the species attract greater forensic interest.

2. Materials and Methods

2.1. Location of Experiments. The experiments were performed in two separate locations:

- (i) Coastal and forest regions of the Juréia-Itatins Ecological Station in the town of Peruíbe in pristine sites unaffected by environmental changes caused by urbanization and located at sea level at 24°22'S latitude and 47°01'W longitude: its predominant vegetation is dense rain forest with humid subtropical climate without dry season. The average annual temperature is 20,5°C and average annual rainfall is recorded in 2277.8 mm.
- (ii) Extremely urbanized region in São Paulo City at a site on the campus of the School of Medicine, University of São Paulo, at an altitude of approximately 868 m at 23°33'S latitude and 46°40'W longitude: the climate is temperately humid with average annual temperature of 18°C and average annual rainfall of 1340 mm.

Ignoring differences in altitude and field routes, the straight-line distance between the two experimental deployment points was approximately 98 km.

2.2. Experimental Model. The experimental model was composed of swine carcasses (*Sus scrofa* Linné, 1758) weighing approximately 12 kg. The animals were previously euthanized by spinal concussion using a pneumatic hammer (humane method), which causes instantaneous death without a great leakage of blood and does not induce chemical changes related to stress that could interfere in the analysis process. This animal model has the highest acceptance rate for studies related to decomposition because the decomposition pattern is similar to that of human body decomposition, the carcasses are relatively easy to obtain and cheap, and the practice does not tend to inspire public objections [5].

Authors have used different names to identify each phase, and in the present study, the division suggested by Catts and Goff that consists of five stages was used [5]: fresh, bloat, active decay, advanced decay, and skeletal remains (skeletonization).

2.3. Experimental Deployment. The carcasses were placed into 70 × 50 × 50 cm cages directly on the ground in approximately 10 cm deep shallow graves. This methodology was applied to protect the carcasses from vertebrate predators and allow adequate colonization by arthropods.

Modified Shannon traps were used as described by Cavallari et al. [16], and they were characterized by a white voile tent fabric properly positioned and fixed at the upper portion (2 m in diameter × 2 m in length) to cover the entire carcass and maintain raised areas above the soil for insect transit and ventilation. The tissue ends were trapped using soil cuttings. This light-colored tent acts to direct insects to the collector bottle, which is located along the upper part, where the insects remain trapped and fixed in alcohol gel until collection. This trap can be deployed indefinitely and provides insect capture over the entire period.

2.4. Experiment Duration. This study was conducted in two stages, with the initial experiments performed in both locations during summer (Jan/2011) and subsequent experiments performed during winter (Aug/2011). Regardless of the duration of carcass decomposition, a thirty-day period for each experiment was implemented to maintain a standard for data collection.

2.5. Collection, Assembly, and Identification. Samples were collected daily during the first two weeks and after this period the collection was made every two days during the last two weeks between 12 pm and 2 pm, a period with an increased photoperiod. The specimens that were captured alive were euthanized with ethyl acetate and placed in 70°GL alcohol for transportation to the laboratory.

The preparation of insects for analysis consisted of washing with distilled water to remove any impurities and traces of alcohol gel. This step was followed by submersion in modified Dietrich's solution, which consists of a solution prepared with distilled water, 95°GL alcohol, formaldehyde, acetic acid, and glycerin. This solution is a special fixative designed to maintain the integrity of stored insects as well as the colors and structures of the specimens [17]. After storage in the solution, the dipterans were pinned and identified at the laboratory using dichotomous keys [17–31].

2.6. Climatic Data. The measurements to obtain climatological data were performed using thermohygrometers (model SH122, J. Prolab Industry and Commerce Products for Laboratory Ltd., São Paulo, SP, Brazil), which were left at the experimental sites and performed daily recordings. The pluviometric indexes were not obtained; however, the maximum and minimum temperature and relative humidity data should be sufficient to correlate the duration of

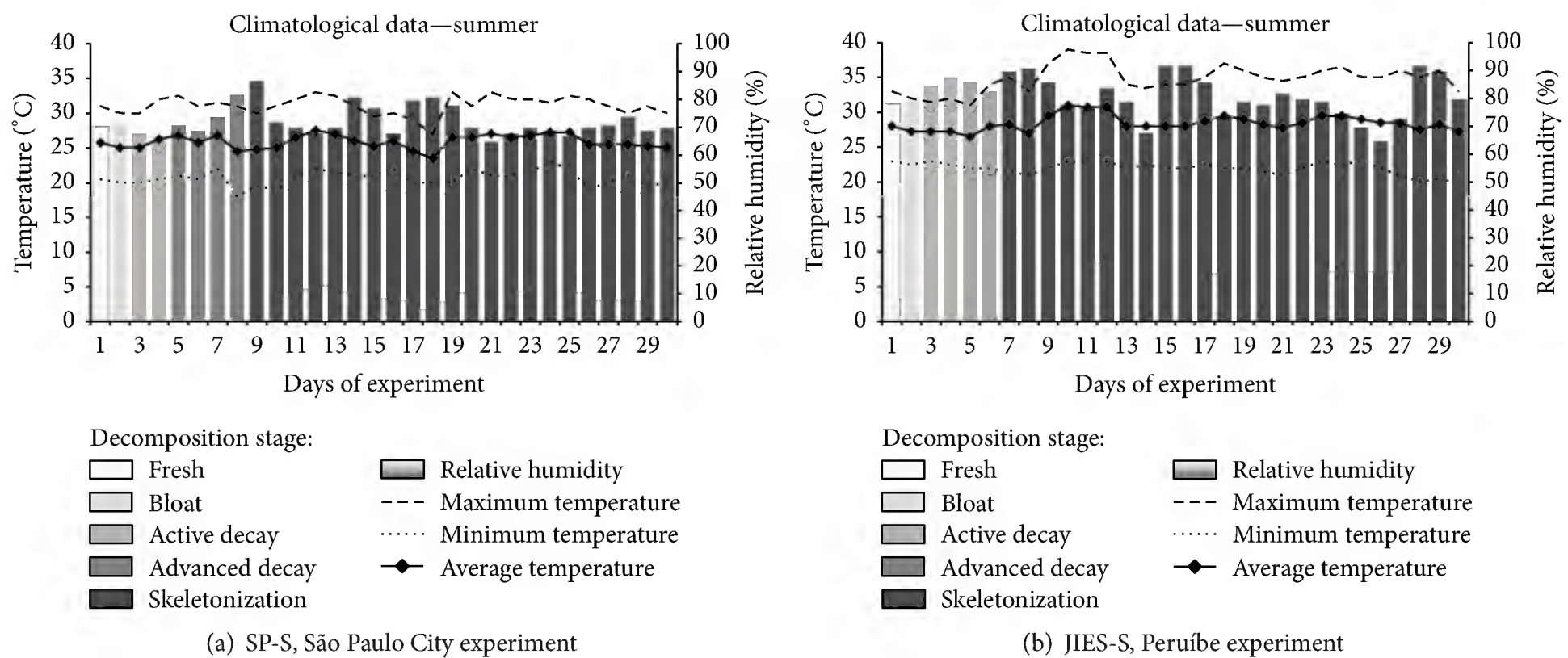


FIGURE 1: Climatological data for the summer experiments.

the decomposition phases with the incidence of the cadaveric fauna succession.

2.7. Statistical Analysis. To identify and compare the fauna from the two different locations, a statistical analysis was performed that included calculating two faunal diversity indexes:

- (i) The Simpson diversity and dominance index, which reflects the probability of two randomly chosen individuals from the same community belonging to the same species.
- (ii) The Shannon-Wiener index, which measures the degree of uncertainty when predicting the species that belong to a randomly chosen specimen: smaller index values indicate a lower degree of uncertainty and represent a sample with low diversity and higher index values indicate a greater degree of uncertainty and represent a sample with greater diversity.

3. Results and Discussion

Two experiments were conducted in each location and season. According to Hanski [32], environmental changes can result in large variations of the species that visit and colonize a carcass. Thus, the insect fauna composition of contrasting habitats must be evaluated at the same location and at different times of the year to establish a forensic entomology baseline that contains information on insect colonization and succession and can be used for future applications and criminal forensic operations [7].

Similar to the methods used by Eberhardt and Elliot [9], the fresh phase was calculated from the moment of swine death until the early bloat phase. As expected, less than a month was required for the carcasses to reach total decomposition, which differs from the results found by Martinez et al. [8], who found that the carcass decomposition

TABLE 1: Number of specimens collected.

Experiment	Collected insects
Summer	
JIES	3123
SP	1705
Winter	
JIES	4095
SP	1572
Total	10495

process required 83 days, and those of Lopes de Carvalho and Linhares [33], who conducted a study during the wet season and found that casting decomposition required 45 days. To verify the decomposition phases and climatological monitoring, a standard 30 days of observation was followed in the present study. However, the collections ended after the skeletonization stage was reached, which was when the last evaluation was conducted. A total of 10,495 specimens (Table 1) were collected, and they were all identified and associated with the decomposition stage at which they were captured.

3.1. Summer Experiments

3.1.1. SP-S. During the experiment in São Paulo City, the average temperature was 25.8°C and the average relative humidity was 72%. The average maximum and minimum temperatures were 31°C and 20.6°C, respectively (Figure 1(a)). The early colonization of the carcass occurred immediately after placement. The weather at placement was hot and humid, which are ideal conditions for the visitation/colonization of a large number of insects. Although storms occurred during the experimental period, especially in the afternoon, decomposition was severe, and on the 9th day, the carcass was already skeletonized. The number of

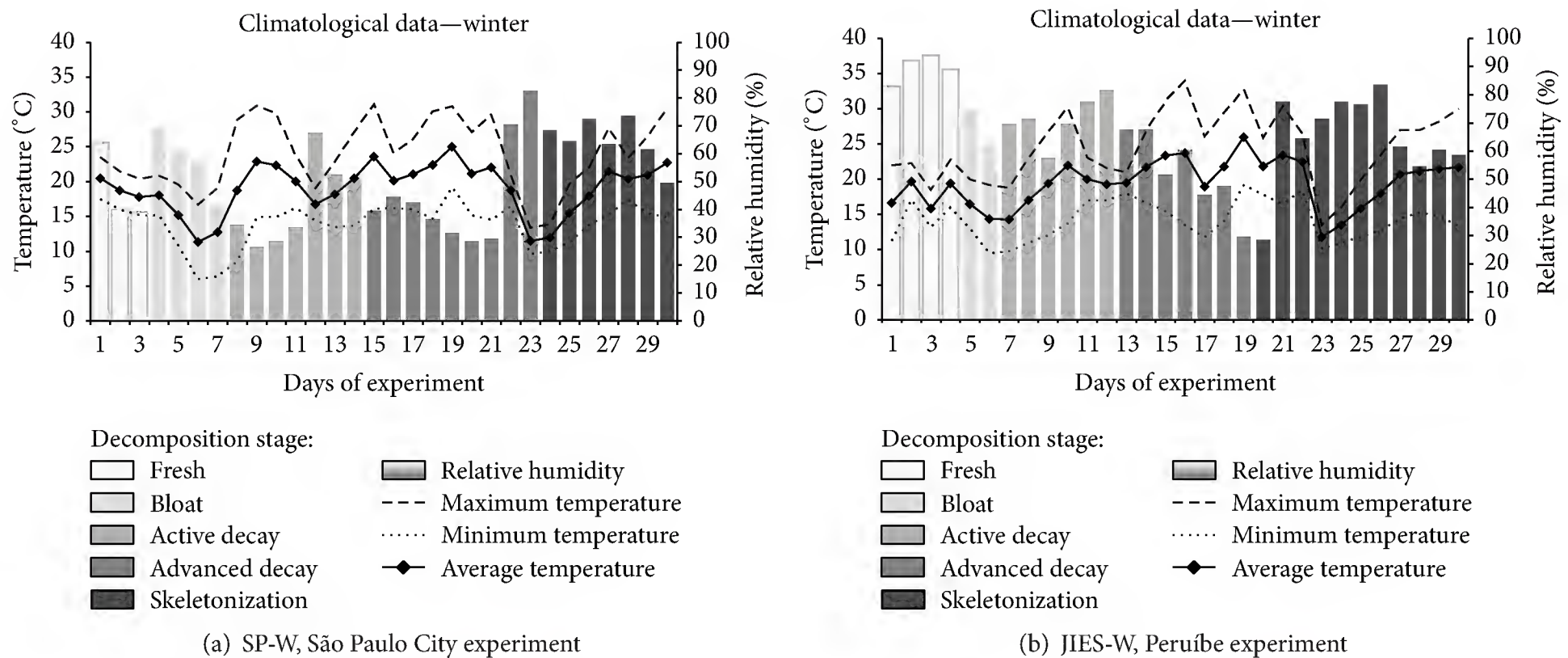


FIGURE 2: Climatological data for the winter experiments.

specimens collected was considered satisfactory and unaffected by the rains during the period of experimentation, which differs from the results found by Eberhardt and Elliot [9], who noted the lowest visitation rates to open-air exposed carcasses and a subsequent deceleration of decomposition during the rainy season. Similar results were published by Lopes de Carvalho and Linhares [33], whose study also coincided with greater rainfall and higher temperatures; thus the decomposition was rapid and intense. The flies were classified as belonging to 11 families, and 27 species were identified from the families with the greatest forensic interest (Calliphoridae, Muscidae, Fanniidae, and Sarcophagidae) (Table 2).

3.1.2. JIES-S. In the experiment inside the Juréia-Itatins Ecological Station in the town of Peruíbe, the average temperature was 28.4°C and the average relative humidity was 81%. The average maximum and minimum temperatures were 34.7°C and 22°C, respectively. The period was characterized by high temperatures and humidity, and the daily maximum recorded temperature exceeded 30°C. Carcass decomposition occurred in 7 days; therefore, it was not possible to differentiate between the bloat and active decay steps among the collections, and these steps were assumed to have occurred on the same day. The collected dipterans were classified as belonging to 16 families, and 39 distinct species were collected from the families with the greatest forensic interest (Table 2).

Figure 1 graphically represents the temperatures and relative humidity measured during the two summer experiments as well as the cadaverous decomposition stages observed during the collection, which were categorized according to changes in the carcasses' appearance [8]. The decomposition stages did not occur in isolation, which is consistent with Freire's [34] assertions that putrefaction stages do not occur simultaneously in all parts of the body or carcass but rather overlap. In the present study, these phases were estimated according to the predominant appearance at the moment of

collection, and the hotter and more humid climates promoted faster decay, which was observed in the experiment at JIES-S. This rapid succession of putrefaction stages can hamper the analysis of cadaverous entomofaunal succession, especially the identification of insects according to the decomposition phase. Therefore, because of rapid putrefaction, the only observations were of the arthropod families or species' preferences for each putrefaction stage; however, the same type of insect may have been present at different phases or even at all phases.

3.2. Winter Experiments

3.2.1. SP-W. At the São Paulo City experimental site, the average temperature was 19.2°C and the average relative humidity was 51%. The average maximum and minimum temperatures were 24.3°C and 14°C, respectively (Figure 2(a)). The low temperatures and humidity likely influenced the period of time required for carcass decomposition because, among the four experiments, this experiment presented the longest time interval until the skeletonization phase. This result corroborates the data published by Wang et al. [35], who reported that, during winter, carcass decomposition was longer relative to other seasons. Among the 19 collected Diptera families, 38 species were collected from the families with the greatest forensic interest (Table 3).

3.2.2. JIES-W. At the experimental site in the town of Peruíbe, the average temperature was 19.5°C and the average relative humidity was 67%. The average maximum and minimum temperatures were 24.8°C and 14.3°C, respectively. The extended range of temperatures and relative humidities during the trial and the sequence of cadaverous decomposition stages over time are shown in Figure 2(b). After observing and comparing the periods of decomposition of the four carcasses during the experiments (Figures 1 and 2), our conclusions are consistent with those of Segura et al.

TABLE 2: Identification of Diptera collected during summer.

Diptera family	Genus/species	São Paulo-summer (SP-S)					Total	Peruíbe-summer (JIES-S)				Total
		Decomposition stage						Decomposition stage				
		F	B	AcD	AdD	S		F	B/AcD	AdD	S	
Anthomyiidae	<i>Hylemyioide plurinervis</i>	—	—	—	—	—	—	1	—	—	—	1
Calliphoridae	<i>Chrysomya albiceps</i>	—	7	12	18	5	42	5	10	9	3	27
	<i>Chrysomya megacephala</i>	—	1	2	—	—	3	2	6	6	—	14
	<i>Chrysomya putoria</i>	—	2	1	5	3	11	—	—	—	—	—
	<i>Cochliomyia macellaria</i>	—	—	—	—	—	—	—	1	—	—	1
	<i>Hemilucilia segmentaria</i>	—	—	—	—	—	—	1	3	3	1	8
	<i>Hemilucilia semidiaphana</i>	—	—	1	—	—	1	12	55	51	48	166
	<i>Lucilia eximia</i>	11	6	9	2	2	30	—	2	1	—	3
	<i>Lucilia purpurascens</i>	—	2	2	1	1	6	5	12	31	10	58
	<i>Paralucilia xanthogeneiates</i>	—	—	—	—	—	—	—	5	4	—	9
Dolichopodidae	sp.	—	—	—	—	—	—	—	—	6	8	14
Drosophilidae	sp.	—	—	—	—	—	—	—	3	5	5	13
Fanniidae	<i>Euryomma carioca</i>	—	5	13	20	8	46	—	—	—	—	—
	<i>Fannia canicularis</i>	—	—	—	1	—	1	—	—	—	—	—
	<i>Fannia femoralis</i>	—	6	9	39	11	65	31	44	117	29	221
	<i>Fannia flavicincta</i>	—	—	—	—	—	—	—	—	5	—	5
	<i>Fannia heydenii</i>	—	—	—	1	1	2	13	24	8	—	45
	<i>Fannia penicilaris</i>	—	—	—	—	—	—	—	1	7	3	11
	<i>Fannia pusio</i>	2	3	3	32	11	51	12	31	109	7	159
	<i>Fannia trimaculata</i>	—	—	—	—	—	—	7	12	52	4	75
	* <i>Fannia</i> sp.	5	10	34	87	47	183	111	215	519	40	885
Lonchaeidae	sp.	—	—	—	—	—	—	—	—	1	—	1
Micropezidae	sp.	2	—	—	—	—	2	1	4	4	3	12
Milichiidae	sp.	—	—	—	—	—	—	—	3	19	68	90
Muscidae	<i>Atherigona orientalis</i>	—	—	—	—	—	—	2	1	8	6	17
	<i>Biopyrellia bipuncta</i>	—	—	—	1	—	1	3	7	2	—	12
	<i>Brontaea delecta</i>	—	—	—	1	—	1	—	—	—	—	—
	<i>Brontaea normata</i>	—	—	1	—	—	1	—	2	—	2	4
	<i>Correntosia bicolor</i>	—	—	—	—	—	—	1	—	—	—	1
	<i>Cyrtoneurina crispaseta</i>	—	—	—	—	—	—	4	12	—	—	16
	<i>Cyrtoneurina</i> sp.	—	—	—	—	—	—	—	1	4	2	7
	<i>Cyrtoneuropsis dubia</i>	—	—	—	—	—	—	9	22	3	1	35
	<i>Cyrtoneuropsis gluta</i>	—	—	—	—	—	—	—	—	—	1	1
	<i>Cyrtoneuropsis maculipennis</i>	—	—	—	—	—	—	1	3	—	—	4
	<i>Cyrtoneuropsis pararescita</i>	—	—	—	—	—	—	—	—	—	2	2
	<i>Cyrtoneuropsis similata</i>	—	—	—	—	—	—	—	—	1	—	1
	<i>Cyrtoneuropsis veniseta</i>	—	—	—	—	—	—	—	6	3	1	10
	<i>Morellia humeralis</i>	—	—	—	—	—	—	2	7	1	—	10
	<i>Musca domestica</i>	1	5	7	4	1	18	3	8	5	—	16
	<i>Mydaea nubivena</i>	—	—	—	—	—	—	1	—	—	—	1
	<i>Neomuscina inflexa</i>	—	—	—	—	—	—	1	—	—	—	1
	<i>Ophyra aenescens</i>	—	9	18	7	7	41	19	29	73	3	124
	<i>Ophyra albuquerquei</i>	—	2	3	—	4	9	—	—	—	—	—
	<i>Ophyra chalcogaster</i>	—	3	8	1	5	17	—	—	—	—	—
	<i>Parapyrellia maculipennis</i>	—	—	—	—	—	—	—	3	2	—	5
	<i>Sarcopromusca pruna</i>	—	—	—	—	—	—	—	1	—	—	1
	<i>Synthesiomysia nudiseta</i>	—	—	1	1	1	3	—	—	—	—	—

TABLE 2: Continued.

Diptera family	Genus/species	São Paulo-summer (SP-S)					Total	Peruíbe-summer (JIES-S)				Total
		Decomposition stage						Decomposition stage				
		F	B	AcD	AdD	S		F	B/AcD	AdD	S	
Otitidae	sp.	—	—	—	3	2	5	30	119	130	76	355
Phoridae	sp.	1	2	1	10	9	23	21	36	124	40	221
Piophilidae	sp.	—	4	4	7	1	16	3	3	17	5	28
Sarcophagidae	<i>Microcerella halli</i>	—	—	—	2	1	3	—	—	7	38	45
	<i>Oxysarcodexia amorosa</i>	—	—	—	—	—	—	1	6	4	1	12
	<i>Oxysarcodexia diana</i>	1	1	5	2	—	9	—	—	—	—	—
	<i>Oxysarcodexia paulistanensis</i>	—	—	—	2	1	3	—	—	—	—	—
	<i>Oxysarcodexia thornax</i>	—	—	3	1	1	5	—	1	—	—	1
	<i>Oxysarcodexia timida</i>	—	—	—	—	—	—	—	1	—	—	1
	<i>Peckia australis</i>	1	—	1	2	1	5	—	—	—	—	—
	<i>Peckia collusor</i>	—	1	1	2	—	4	—	—	—	—	—
	<i>Peckia intermutans</i>	—	—	—	—	—	—	—	2	—	—	2
	<i>Ravinia belforti</i>	—	1	3	2	1	7	—	—	—	—	—
	<i>Sarcodexia lambens</i>	—	—	4	2	1	7	—	—	—	1	1
	**sp.	—	3	22	18	6	49	1	6	8	41	56
Sepsidae	sp.	10	200	208	424	189	1031	20	26	118	106	270
Stratiomyidae	<i>Auloceromyia</i> sp.	—	—	—	—	—	—	1	1	—	—	2
	<i>Hermetia illucens</i>	—	—	—	—	—	—	3	5	9	9	26
	<i>Hermetia</i> sp.	—	—	—	—	—	—	—	—	1	1	2
	<i>Merosargus</i> sp.	—	—	—	—	—	—	—	—	2	4	6
	<i>Sargus</i> sp.	—	—	—	—	—	—	—	1	—	—	1
Syrphidae	<i>Eristalis transversa</i>	—	—	—	—	—	—	—	—	2	1	3
	<i>Ornidia obesa</i>	—	—	2	1	—	3	—	2	—	—	2
	sp.	—	—	—	—	—	—	—	1	2	—	3
Tephritidae	sp.	1	—	—	—	—	1	—	—	—	—	—
Total of collected insects		35	273	378	699	320	1705	327	743	1483	570	3123

F: fresh; B: bloat; AcD: active decay; AdD: advanced decay; S: skeletonization.
* *Fannia* sp.: female specimens belonging to the *pusio* subgroup.
** sp.: females specimens not identified to a genus or species.

[36], who stated that the duration of the decay process is dependent on the particular environmental conditions as well as the activity of the insects associated with the carcass. Of the 4,095 flies collected, 35 Diptera families were identified, and 73 species were collected from the families with the greatest forensic interest (Table 3).

Variations in the insects collected throughout the study are shown in Figure 3, and the highest visitation period to the carcasses occurred during the phases of active and advanced decay, which was also noted by Valdes-Perezgasga et al. [37]. In both seasons, visitations were greater at the dense forest location compared with that of the urban area, which indicates that there are greater numbers of dipterous insects in the forest regions relative to the amount of effectively synanthropic insects.

3.3. *Faunistic Indexes.* The Simpson diversity and dominance index (Figure 4) indicates that diversity and dominance are inversely proportional [38]; thus, diversity tends to increase and dominance tends to decrease during active and advanced

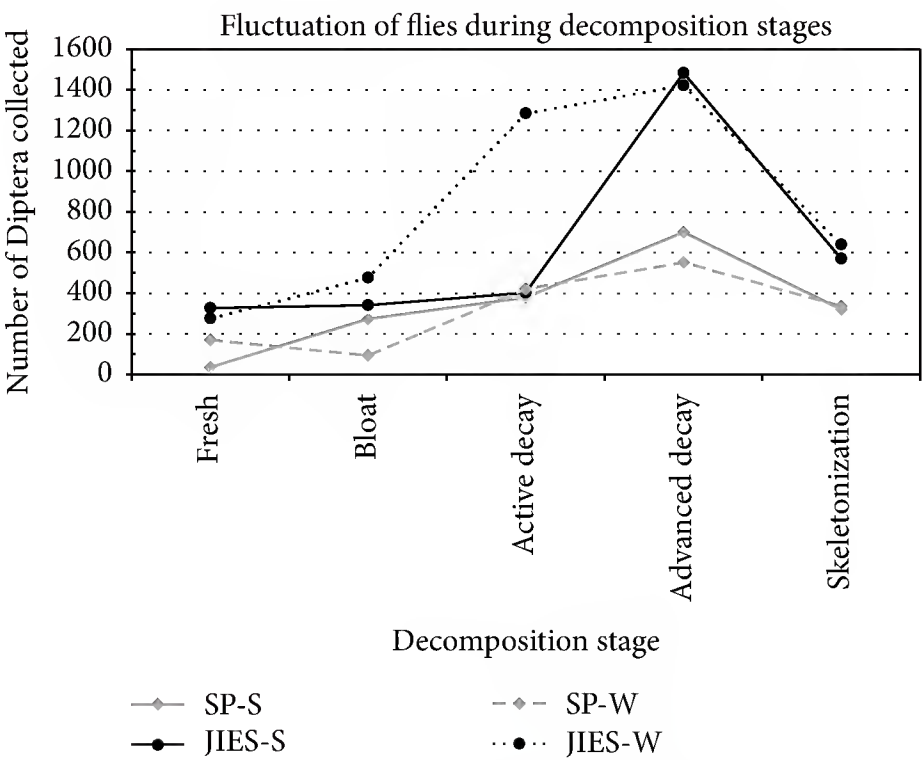


FIGURE 3: Graphical comparison of dipteran fluctuations. SP-S: summer experiment, São Paulo. SP-W: winter experiment, São Paulo. JIES-S: summer experiment, Perúibe. JIES-W: winter experiment, Perúibe.

TABLE 3: Identification of Diptera collected during winter.

Diptera family	Genus/species	São Paulo-winter (SP-W)						Peruíbe-winter (JIES-W)					
		Decomposition stage					Total	Decomposition stage					Total
		F	B	AcD	AdD	S		F	B	AcD	AdD	S	
Anisopodidae	sp.	—	—	—	—	—	—	—	—	—	—	1	1
Anthomyiidae	<i>Anthomyia</i> sp.	3	2	—	1	—	6	—	—	—	—	—	—
Apioceridae	sp.	—	—	—	—	—	—	—	—	—	—	1	1
Calliphoridae	<i>Chrysomya albiceps</i>	—	—	34	138	77	249	—	2	2	3	2	9
	<i>Chrysomya megacephala</i>	—	—	1	9	5	15	—	—	—	—	—	—
	<i>Chrysomya putoria</i>	—	—	8	28	14	50	—	—	—	—	—	—
	<i>Cochliomyia macellaria</i>	—	—	—	1	—	1	—	—	—	—	1	1
	<i>Compsomyiops fulvicrura</i>	—	—	—	—	—	—	4	3	5	1	—	13
	<i>Hemilucilia segmentaria</i>	—	—	—	—	—	—	1	—	17	4	5	27
	<i>Hemilucilia semidiaphana</i>	—	—	—	—	—	—	5	4	33	48	15	105
	<i>Lucilia cuprina</i>	—	—	—	—	1	1	—	—	—	—	—	—
	<i>Lucilia eximia</i>	38	22	31	21	8	120	9	1	—	—	—	10
	<i>Lucilia purpurascens</i>	102	47	94	87	33	363	1	—	4	5	1	11
	<i>Lucilia</i> sp.	—	2	4	1	—	7	—	—	—	—	—	—
	<i>Mesembrinella bellardiana</i>	—	—	—	—	—	—	—	1	6	3	4	14
	<i>Mesembrinella</i> sp.	—	—	—	—	—	—	—	—	2	4	3	9
	<i>Paralucilia fulvinota</i>	—	—	—	—	—	—	—	—	29	20	6	55
	<i>Paralucilia xanthogeneiates</i>	—	—	—	—	—	—	22	84	214	165	18	503
Ceratopogonidae	sp.	—	—	—	—	—	—	1	—	—	—	—	1
Chloropidae	sp.	—	—	12	2	3	17	—	3	8	34	26	71
Chyromyidae	sp.	—	—	—	—	—	—	—	1	—	—	—	1
Clusiidae	sp.	—	—	—	—	—	—	1	—	—	—	—	1
Conopidae	sp.	—	—	—	—	—	—	1	—	—	—	—	1
Culicidae	sp.	—	—	—	—	—	—	1	—	—	—	—	1
Dolichopodidae	sp.	—	—	—	—	—	—	—	1	9	11	8	29
Drosophilidae	sp.	1	4	37	13	24	79	3	10	18	42	18	91
Empididae	sp.	—	—	—	—	—	—	—	—	—	5	—	5
Fanniidae	<i>Euryomma carioca</i>	—	3	3	7	2	15	—	—	1	1	1	3
	<i>Euryomma peregrinum</i>	—	—	—	—	—	—	—	—	—	1	1	2
	<i>Fannia canicularis</i>	—	—	—	—	—	—	2	—	—	—	—	2
	<i>Fannia femoralis</i>	—	—	6	7	2	15	1	10	13	9	3	36
	<i>Fannia flavicincta</i>	—	—	—	—	—	—	1	1	4	12	5	23
	<i>Fannia heydenii</i>	—	—	1	—	—	1	12	30	109	122	22	295
	<i>Fannia obscurinervis</i>	—	—	—	—	—	—	—	—	20	15	3	38
	<i>Fannia penicilaris</i>	—	—	—	—	—	—	—	—	—	59	21	80
	<i>Fannia punctipennis</i>	—	—	—	—	—	—	1	—	—	—	—	1
	<i>Fannia pusio</i>	—	—	—	1	1	2	—	1	5	1	1	8
	<i>Fannia sabroskyi</i>	—	—	—	4	—	4	—	—	—	2	—	2
	<i>Fannia snyderi</i>	—	—	—	—	—	—	—	—	—	5	2	7
	<i>Fannia trimaculata</i>	—	—	—	2	1	3	—	—	1	5	3	9
	<i>Fannia yenhedi</i>	—	—	—	—	—	—	—	—	—	6	1	7
	* <i>Fannia</i> sp.	6	—	45	65	40	156	27	77	126	199	65	494
Heleomyzidae	sp.	—	—	—	1	—	1	—	—	—	—	—	—
Lauxaniidae	sp.	—	—	—	—	—	—	1	1	4	—	—	6
Lonchaeidae	sp.	—	—	—	—	—	—	1	—	3	16	8	28
Micropezidae	sp.	—	—	—	—	—	—	15	7	35	24	33	114
Milichiidae	sp.	—	1	1	4	—	6	—	9	33	10	29	81

TABLE 3: Continued.

Diptera family	Genus/species	São Paulo-winter (SP-W)					Total	Peruíbe-winter (JIES-W)					Total
		Decomposition stage						Decomposition stage					
		F	B	AcD	AdD	S		F	B	AcD	AdD	S	
Muscidae	<i>Atherigona orientalis</i>	—	—	1	—	—	1	—	—	—	1	—	1
	<i>Biopyrellia bipuncta</i>	—	—	—	—	—	—	1	—	—	2	—	3
	<i>Brontaea debilis</i>	—	—	—	—	—	—	—	1	—	—	—	1
	<i>Brontaea delecta</i>	—	—	—	1	—	1	—	2	—	—	—	2
	<i>Brontaea normata</i>	—	—	2	—	—	2	1	—	—	—	—	1
	<i>Brontaea</i> sp.	—	—	—	—	—	—	—	—	—	—	1	1
	<i>Cyrtoneurina</i> cf. <i>alifusca</i>	—	—	—	—	—	—	—	—	1	3	1	5
	<i>Cyrtoneurina</i> sp.	—	—	—	—	—	—	3	2	3	18	5	31
	<i>Cyrtoneurina varicolor</i>	—	—	—	—	—	—	—	—	—	1	—	1
	<i>Cyrtoneuropsis dubia</i>	—	—	—	—	—	—	19	23	13	36	4	95
	<i>Cyrtoneuropsis incognita</i>	—	—	—	—	—	—	—	—	1	1	1	3
	<i>Cyrtoneuropsis maculipennis</i>	—	—	—	—	—	—	—	—	—	24	8	32
	<i>Cyrtoneuropsis varicolor</i>	—	—	—	—	—	—	—	—	19	10	—	29
	<i>Cyrtoneuropsis veniseta</i>	—	—	—	—	—	—	2	4	8	19	4	37
	<i>Graphomya analis</i>	—	—	—	1	—	1	—	—	—	—	—	—
	<i>Limnophora deleta</i>	—	—	—	—	—	—	—	—	1	2	1	4
	<i>Morellia humeralis</i>	—	—	—	—	—	—	1	—	—	—	—	1
	<i>Morellia violacea</i>	—	—	—	—	—	—	1	—	—	—	—	1
	<i>Musca domestica</i>	—	—	2	1	2	5	—	—	—	—	—	—
	<i>Muscina stabulans</i>	—	1	1	1	1	4	—	—	—	—	—	—
	<i>Mydaea nubivena</i>	—	—	—	—	—	—	—	—	—	—	1	1
	<i>Mydaea plaumanni</i>	1	—	—	—	—	1	—	—	—	—	1	1
	<i>Myospila fluminensis</i>	—	—	2	—	—	2	—	—	2	—	—	2
	<i>Neomuscina currani</i>	—	—	—	—	—	—	—	—	2	4	—	6
	<i>Neomuscina inflexa</i>	—	—	—	—	—	—	—	—	1	1	—	2
	<i>Neomuscina</i> sp.	—	—	—	—	—	—	1	—	—	—	—	1
	<i>Neomuscina tinctinervis</i>	—	—	—	—	—	—	—	—	—	2	—	2
	<i>Ophyra aenescens</i>	—	—	5	63	37	105	4	60	31	21	15	131
	<i>Ophyra albuquerquei</i>	—	—	1	2	2	5	—	3	3	3	3	12
	<i>Ophyra capensis</i>	—	—	—	10	2	12	—	—	—	—	—	—
	<i>Ophyra chalcogaster</i>	—	1	3	3	—	7	—	—	1	1	1	3
	<i>Ophyra solitaria</i>	—	—	—	—	—	—	2	3	3	3	2	13
	<i>Ophyra</i> sp.	—	—	—	2	—	2	—	—	—	—	—	—
	<i>Phaonia</i> sp.	—	—	—	—	—	—	—	1	—	—	—	1
	<i>Pseudoptilolepis</i>	—	—	—	—	—	—	—	—	5	3	—	8
	<i>Synthesiomyia nudiseta</i>	10	2	35	—	8	55	2	2	7	1	2	14
Mycetophilidae	sp.	—	1	—	—	—	1	—	—	1	5	1	7
Neriidae	sp.	—	—	—	1	—	1	—	—	—	1	1	2
Otitidae	sp.	—	—	8	3	12	23	15	24	77	9	3	128
Phoridae	sp.	5	3	34	20	21	83	54	53	191	215	140	653
Piophilidae	sp.	—	—	3	3	9	15	1	—	1	13	34	49
Pompilidae	sp.	—	—	—	—	—	—	—	—	—	1	—	1
Psilidae	sp.	—	—	—	6	—	6	—	—	—	—	—	—
Richardiidae	sp.	—	—	—	—	—	—	—	—	2	—	—	2
Ropalomeridae	sp.	—	—	—	—	—	—	—	—	—	—	2	2

TABLE 3: Continued.

Diptera family	Genus/species	São Paulo-winter (SP-W)						Peruíbe-winter (JIES-W)						
		Decomposition stage					Total	Decomposition stage					Total	
		F	B	AcD	AdD	S		F	B	AcD	AdD	S		
Sarcophagidae	<i>Boettcheria aurifera</i>	—	—	—	—	—	—	2	—	—	—	—	—	2
	<i>Engelimyia inops</i>	—	—	—	—	—	—	—	—	—	1	—	1	
	<i>Helicobia aurescens</i>	—	—	1	—	—	1	—	—	—	—	—	—	
	<i>Microcerella halli</i>	—	—	1	4	—	5	—	—	—	2	—	2	
	<i>Oxysarcodexia admixta</i>	—	—	—	1	—	1	—	—	—	1	—	1	
	<i>Oxysarcodexia amorosa</i>	—	—	—	—	—	—	—	2	3	2	2	9	
	<i>Oxysarcodexia angrensis</i>	—	—	—	—	—	—	—	—	1	—	—	1	
	<i>Oxysarcodexia culmiforceps</i>	—	—	—	—	—	—	—	—	1	—	—	1	
	<i>Oxysarcodexia diana</i>	—	—	2	1	—	3	—	—	3	3	4	10	
	<i>Oxysarcodexia fluminensis</i>	—	—	—	—	—	—	—	—	1	—	—	1	
	<i>Oxysarcodexia paulistanensis</i>	—	—	2	3	—	5	—	—	—	—	—	—	
	<i>Oxysarcodexia thornax</i>	—	—	1	4	—	5	—	—	—	—	—	—	
	<i>Oxysarcodexia timida</i>	—	—	—	—	—	—	—	—	—	—	2	2	
	<i>Oxysarcodexia xanthosoma</i>	—	—	—	—	—	—	—	—	—	—	1	1	
	<i>Peckia anguilla</i>	—	—	—	—	—	—	—	—	1	1	—	2	
	<i>Peckia australis</i>	—	—	2	—	—	2	—	—	—	—	—	—	
	<i>Peckia chrysostoma</i>	—	—	—	—	—	—	—	—	1	—	—	1	
	<i>Peckia collusor</i>	—	—	1	—	—	1	—	—	1	—	—	1	
	<i>Peckia intermutans</i>	—	—	—	—	—	—	—	—	1	2	—	3	
	<i>Ravinia belforti</i>	—	—	1	—	—	1	—	—	1	1	—	2	
	<i>Sarcodexia lambens</i>	—	—	—	—	—	—	—	—	2	—	1	3	
	<i>Titanogrypa fimbriata</i>	—	—	1	1	—	2	—	—	—	—	—	—	
		** sp.	3	5	16	21	23	68	13	16	47	22	24	122
Sciaridae	sp.	1	—	—	—	—	1	5	3	—	—	—	8	
Sepsidae	sp.	—	—	17	4	8	29	28	28	130	128	50	364	
Sphaeroceridae	sp.	—	—	2	2	—	4	—	1	—	5	8	14	
Stratiomyidae	<i>Sargus</i> sp.	—	—	—	—	—	—	1	—	—	—	2	3	
Strongylophthalmyiidae	sp.	—	—	—	—	—	—	1	—	—	—	—	1	
Syrphidae	<i>Ornidia obesa</i>	—	—	—	—	—	—	—	—	—	—	1	1	
	<i>Pyrirtis</i> sp.	—	—	—	—	—	—	1	—	—	—	—	1	
	<i>Rhingia</i> sp.	—	—	—	—	—	—	2	—	—	—	—	2	
	<i>Syrphus</i> sp.	—	—	—	—	—	—	5	—	3	4	2	14	
	sp.	—	—	—	1	—	1	—	—	1	—	1	2	
Tethinidae	sp.	—	—	—	—	—	—	—	—	5	18	3	26	
Tipulidae	sp.	—	—	—	—	—	—	—	2	7	—	—	9	
Total of collected insects		170	94	421	551	336	1572	275	476	1283	1422	639	4095	

F: fresh; B: bloat; AcD: active decay; AdD: advanced decay; S: skeletonization.
* *Fannia* sp.: female specimens belonging to the *pusio* subgroup.
* * sp.: females specimens not identified to a genus or species.

decay phases. These results differ from those reported by Al-Mesbah et al. [39], who concluded that species variations decline over time, although, after 10 days, the population was relatively constant, a state that may have been influenced by habitat differences and the animal model used (rabbit).
The Shannon-Wiener diversity index was calculated according to the experimental location: SP-S ($H' = 0.7543$), JIES-S ($H' = 1.1758$), SP-W ($H' = 1.1728$), and JIES-N ($H' = 1.3610$). The highest index corresponds to

the experiment with the greatest species richness (JIES-W) and the largest number of collected insects, thus confirming the results obtained by Magurran [40], who assigned the highest index to the experiment with the highest number of identified species. The index values of the experiments at SP-W and JIES-S were similar, which shows that, despite differences in the number of collected specimens (1,572 and 3,123, resp.), the similar species richness values (55 and 59) approach the index value [38]. The lowest species richness

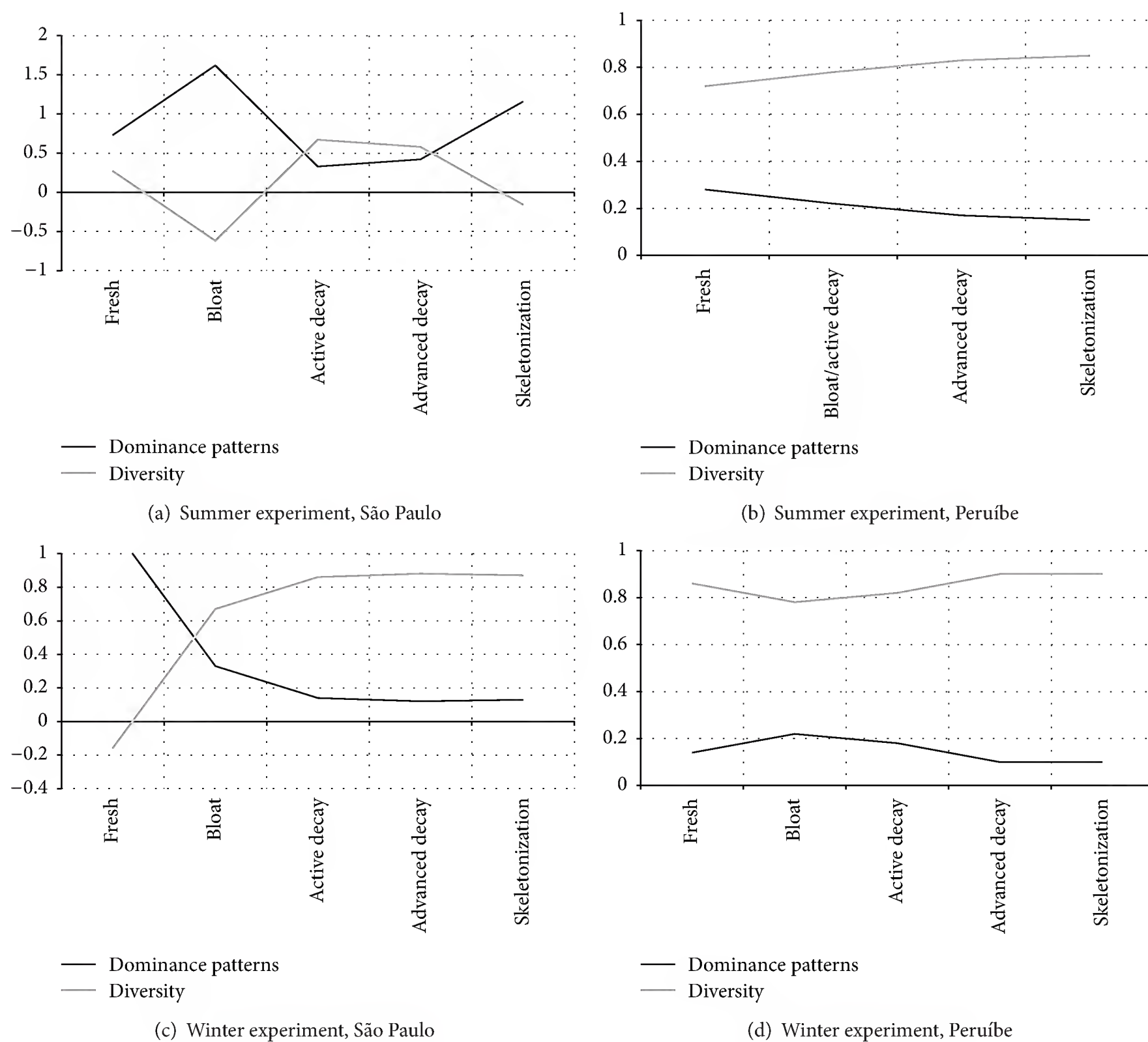


FIGURE 4: Graphical comparison of the Simpson dominance and diversity indexes.

was found in experiment SP-S, which also had the lowest Shannon-Wiener index, indicating that the most common species tend to have higher population levels and rare species have lower population levels [41].

3.4. Diptera. The analyses of the results obtained in the four experiments show that a large number of insects belonging to Calliphoridae, Muscidae, Fanniidae, and Sarcophagidae families visited all carcasses; however, these families did not show the highest visitation frequencies in all of the trials. Sarcophagidae, for example, were not always among the most abundant ones. Specimens belonging to the families Phoridae, Sepsidae, Otitidae, and Piophilidae appeared in all of the experiments and were abundant at times and less frequent at other times but always present. The same trend occurred for the families with forensic interest cited above (Figure 5). Several studies have reported correlations among at least one of these families [6–8, 11, 33, 35] and cadaverous decomposition. The forensic significance of these insects is

likely related to their characteristics as local counters or indicators for different decomposition stages or the PMI.

The succession pattern of families observed in the samples during the decomposition process was similar to the results of the studies by Martinez et al. [8] and Tabor et al. [42]; however, significant variation occurred among the species. Compared with the results from studies performed in Brazil [6, 33], in which species such as *Chrysomya albiceps*, *Chrysomya putoria*, *Chrysomya megacephala*, *Lucilia eximia*, *Hemilucilia segmentaria*, *Hemilucilia semidiaphana*, *Cochliomyia macellaria*, *Mesembrinella bellardiana*, *Oxysarcodexia riograndensis*, *Peckia intermutans*, and *Ravinia belforti* were cited, greater species richness was observed in the present study.

Lopes de Carvalho and Linhares [33] considered *H. segmentaria* and *H. semidiaphana* to be forest area indicators; however, in the present study, a specimen was collected in the urban area, which suggests synanthropy in these insects because they can easily adapt to different environments as long as the necessary substrates for feeding and reproduction

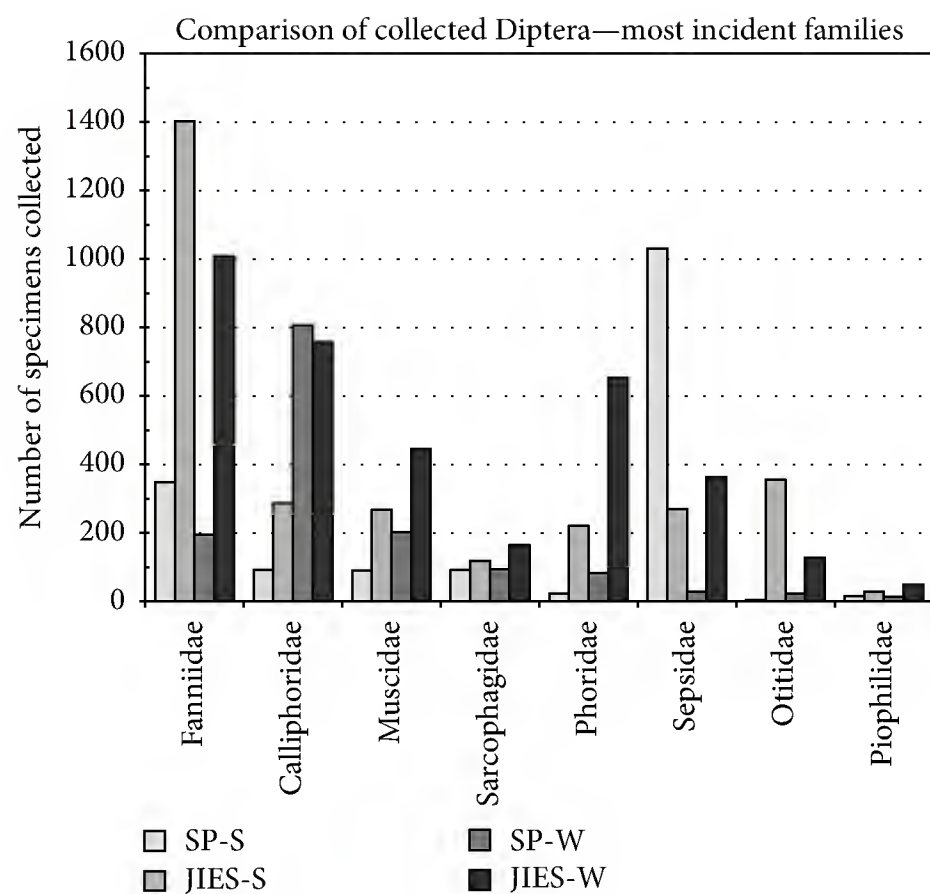


FIGURE 5: Graphical comparison of the collected Diptera from the families of forensic interest. SP-S: summer experiment, São Paulo. SP-W: winter experiment, São Paulo. JIES-S: summer experiment, Peruíbe. JIES-W: winter experiment, Peruíbe.

are present. *Cyrtoneurina* sp. and *Cyrtoneuropsis* sp. and two species of genus *Paralucilia* were reported in this study, and they can be considered forest indicators because they were only observed in the forest region. Moreover, species of the Stratiomyidae family were only found in the experiments located in the Juréia-Itatins Ecological Station, whereas *C. putoria* was only found in the urban areas, which indicates that it has adapted to coexist among people.

Fanniidae species were found in both locations, although a greater number of species from the genus *Fannia* were found in the forest region, whereas a greater number of species from the genus *Euryomma* were observed in the urban areas. Sarcophagidae specimens were found in all of the experiments; however, a greater specific variety was observed in the urban areas during summer, although a relatively small number of insects were found. This result corroborates the data published by Lopes de Carvalho and Linhares [33], who also found a large number of species represented by few specimens visiting the carcass.

4. Conclusions

The results obtained in the present study indicate that seasonal variations and differences in the environment influence the cadaverous decomposition process and the insects that colonize the carcass and their succession. The difference between putrefaction stages led to large variations in the number of attracted insects, and this result confirms the predilection of these insects for consuming resources offered by the carcass in each decomposition stage. Specimens belonging to the families Phoridae, Sepsidae, Otitidae, and Piophilidae were found in all of the experiments, which

suggests greater forensic importance for these insects. Thus, it is recommended to conduct studies that emphasize these families to expand the range of available insects as forensic indicators, which would be beneficial for criminal forensic investigations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Morphometric Analysis of the Host Effect on Phenotypical Variation of *Belminus ferroae* (Hemiptera: Triatominae)

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The Triatominae subfamily includes hematophagous insects, well known for their role as vectors for the *Trypanosoma cruzi* parasite, etiologic agent of Chagas’ disease. *Belminus ferroae* is a triatomine that showed an increased demographic fitness when cockroaches were used as hosts. Here we compare the centroid size (CS) and wing shape between *B. ferroae* parents and three successive generations (O1, O2, and O3) of their offspring fed on cockroaches or mice under laboratory conditions. Morphometric analysis of the wings bugs fed on cockroaches showed a significant reduction in CS in both sexes among all generations. Sexual size dimorphism (SSD) was observed in the insects fed on cockroaches (O2 and O3), as well as those bugs fed on mice (O2). Differences in the shape of wings were observed between parental and offspring wings when fed on mice, but not in males (O1, O2, and O3) or females (O1 and O2) fed on cockroaches. There was a greater wing shape similarity between the cockroach-fed offspring and their parents according to the Mahalanobis distances. Our results support the idea of higher adaptation of this Triatominae with arthropod hosts.

1. Introduction

The Triatominae are true bugs comprising a subfamily of Reduviidae (Hemiptera: Heteroptera) with 147 species [1]. The members of this subfamily are almost entirely hematophagous, although some species feed on other invertebrates [2, 3]. The genus *Belminus* belongs to this taxonomic group comprised of eight species found in Mexico, Costa Rica, Panama, Colombia, Venezuela, Peru, and Brazil [4]. Their feeding behavior includes cannibalism, cleptohematophagy, hemolymphagy, and hematophagy [5–8].

It has been suggested that arthropods could be principal food sources of *Belminus ferroae* and cockroaches have

been implicated as probable hosts within human dwellings [4]. Recently comparison of cohorts of this species fed on arthropods (*Blaberus* and *R. prolixus*) and vertebrates (mice) showed for the first time the existence of a triatomine not restricted to bloodsucking. Moreover, feeding on cockroaches offered clear demographic advantages, namely, higher intrinsic rate of natural increase, finite rate of population growth, and net reproductive rate [2].

Our current study analyzes *Belminus* form by measuring variations of wing shape and centroid size between *B. ferroae* individuals captured in human dwellings and three successive offspring generations reared under laboratory conditions. By associating changes in these variables (i.e., wing centroid

size and shape) with the different hosts, we evaluate the premise that adaptation to a food source produces greater morphologic similarity (of size and shape) between parents and their offspring. Although this is our principal hypothesis, alternative explanations are also discussed to account for unexpected results. Among other analytic tools used, geometric morphometry was chosen for the usefulness it was shown to have in identifying the habitat origin of reinfesting triatomines [9] and in detecting the effects of hosts in a variety of insect groups [10, 11].

2. Materials and Methods

2.1. Insects. The parental insects used in this study were captured in dwellings located in Toledo municipality of the Department of Norte de Santander, Colombia. The region is characterized by a humid premontane forest landscape, with a range of altitudes between 1190 and 1499 m.a.s.l., an average annual temperature of 25°C, and a relative humidity of 85% [4].

2.2. Maintenance of the Parental Insects. A total of 35 individuals (14 males and 21 females), captured in the field, were organized in eight containers, putting one or two males and two or three females in each. These containers were divided into two groups, four belonging to the group fed on mice and four belonging to the group fed on cockroaches. In the mice-fed group, the insects were fed on sedated (Ketalar 75 mg/kg) hosts for 3 h once a week. The cockroach-fed group was maintained with adults of *Blaberus giganteus*. The colony of cockroaches was founded from specimens collected from the same area as the *B. ferroae*. They were replaced monthly and maintained with a mixture of rabbit food, corn, and moist cotton. Plastic containers of 10 × 7 × 2 cm with absorbent paper inside were used to raise the insects; the lids covering the containers were perforated to prevent water condensation and mold.

2.3. Maintenance of Offspring. Containers with parental insects were checked weekly to extract and transfer the eggs to new breeding recipients at a density of 10 insects per container, until adults emerged as offspring 1 (O1). Then, random couples were organized, one to each of 30 containers. Fifteen couples were fed on mice and fifteen on cockroaches, and their eggs gave rise to offspring 2 (O2), and this process continued until offspring 3 (O3). The feeding scheme for offspring fed on mice was similar to that used for the parents. For offspring fed on cockroaches, the first, second, and third instar nymphs of *B. ferroae* were kept with nymphs of *B. giganteus* (fourth or fifth instar), and the elder instars were kept with an adult cockroach.

The experimental breeding was carried out in an acclimatized cabin with constant temperature (25 ± 2°C), relative humidity (70 ± 10% RH), and photoperiod (12:12 h light/dark). The procedures of animal management were carried out in accordance with the Colombian law (National Animal Protection Statute, Law 84, 1989, and Resolution 8430, 1993, of the Colombian Health Ministry).

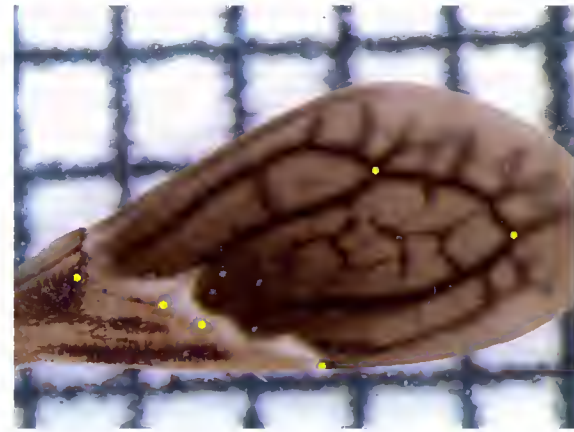


FIGURE 1: Six landmark positions on the left wing of *B. ferroae* used in this study.

2.4. Wing Mounting. Both fore wings of each specimen were removed and mounted in a dorsal position on a slide under a stereoscopic microscope (M205 C, Leica). The wings were mounted between a microscope slide and a cover slip adhered by its edges with liquid silicone. Each specimen was assigned a code which was registered for each slide. Relative data (i.e., date of mounting, number of wings, sex, and code of breeding container) were saved on a spreadsheet.

2.5. Image Capture and Digitalization. A total of 590 left wings were photographed with a CMOS camera (EC3, Leica) coupled to the stereoscope, each one three times. Six landmarks were chosen and all belonged to *type 1* [12] (see Figure 1). The photographs were exported and landmarks were digitized with the software tpsDig (version 2.7) to obtain the geometric coordinates. Preparation of the input files to tpsDig as well as for file organization and conversions afterwards was performed using the software tpsUtil (version 1.47).

2.6. Error Estimates. To reduce the error in the digitalization of the landmarks, this work was done by a single person. The error was estimated by means of the index of repeatability, as described by Arnqvist and Mårtensson [13], which uses a one-way analysis of variance (ANOVA) with repeated measurements, where “*R*” indicates the quotient between the individual variation and the total variance [14]. Parents and their corresponding O1 reared on cockroaches or mice were included in this analysis. The test was carried out with VAR software on each sex separately.

2.7. Size Variation. For comparison of the overall wing size between parents and offspring, the isometric estimator known as “centroid size” (CS) was used, which is defined as the square root of the sum of the squared distances between the centroid of the configuration of landmarks and each individual landmark [15]. The CS was calculated using a package program for doing geometric morphometrics (MorphoJ 1.05e software) [16]. Sexual size dimorphism (SSD) and metric changes in CS were analyzed for each sex using the Kruskal-Wallis test, and the statistical significance was adjusted according to the Bonferroni correction (PAST 3.01 software).

2.8. Shape Variation. The shape variables (Procrustes coordinates) were obtained using the MorphoJ program, which

uses the generalized Procrustes analysis (GPA) that is a superimposing algorithm [16]. Nonetheless, since the number of variables exceeds the number of degrees of freedom, the Procrustes coordinates cannot be used as standard variables in multivariate statistical tests. For this reason a nonparametric alternative involving resampling procedures as bootstrap and permutation was necessary [12, 17]. These analyses do not demand estimation of the degrees of freedom.

In order to compare the variations of shape between the parents captured in the field and the offspring reared in the laboratory with the different hosts, a nonparametric MANOVA (NPMANOVA) was carried out using statistical software (PAST 3.01). Then, canonical variate analysis (CVA), which computes the Mahalanobis distances based on all the groups incorporated in the study, was performed (MorphoJ software). Additionally, a discriminant analysis (DA) was executed to compare each pair of groups separately and to calculate percentages of reclassification and cross-checking. All tests were carried out with 10,000 permutations and the statistical significance was adjusted according to the Bonferroni correction [12, 16].

2.9. Allometry. The relationship between CS and shape variation was examined by multivariate regression (MorphoJ software). The statistical significance of this test was estimated by permutations using 10,000 runs [16].

2.10. Size Correction. The procedure proposed by Viscosi and Cardini [18] was used to verify if wing shape differences were due only to variations in size, or if the variability of shape was affected by the feeding treatments. This method consisted in examining the differences in the allometric pattern (i.e., the slopes) on all offsprings by sex. These differences were analyzed using a full multivariate covariance analysis (MANCOVA) model, with “groups” as the predictor factor and “CS” as the covariate (SPSS 19 software). The interaction term $group \times CS$ was used to evaluate the differences between the slopes of the allometric trajectories between different groups of offsprings [12]. The statistical significance was estimated using 1,000 bootstraps (SPSS 19 software).

In those groups, where it was clear that no significant differences existed in the allometric patterns, the correction for size was carried out by means of a pooled regression within subgroups O1, O2, and O3; shape was used as dependent variable and CS as covariate (MorphoJ). This allowed removal of the within-offspring allometric variation and computation of the regression residuals subjected to other “after-correction” statistical analyses (i.e., NPMANOVA, CVA, and DA).

2.11. Visualizing Shape Variation. An analysis of shape variation was performed using a computer equipped with software (MorphoJ). A wireframe is a set of lines (wires) connecting the digitized points (landmarks) on a shape. The soft wireframe graphs show shifts of the landmarks from the starting to the target shape [19]. The first wireframe was drawn on the starting shape (in our case, the shape parent is shown in light blue; see Figures 4 and 5). A second wireframe is drawn on the target shape, which in this study corresponds to the

offspring shape (O1, O2, and O3) and permits seeing the result of the deformation caused on the starting shape (dark blue, see Figures 4 and 5). The vertices of the wireframes show where the landmarks of the two shapes differ in location. If the wires in the target shape appear as curves, this reflects the deformation of the space between landmarks as inferred from the techniques employed with the thin-plate spline [20]. To better represent the shape change, the scale factor was increased fivefold [18].

2.12. Program Sources. tpsDig2 and tpsUtil are to be found in <http://life.bio.sunysb.edu/morph/>, MorphoJ in http://www.flywings.org.uk/morphoj_page.htm, PAST in <http://folk.uio.no/ohammer/past/>, and VAR in <http://mome-clic.com/clic-collection/clicmodules/var>.

3. Results

3.1. Error Estimation. Comparison of three repeated sets of photographs of the same set of wings showed fairly good agreement for the centroid size on females ($n = 94$, $R = 0.992$, 0.993 , and 0.992) and males ($n = 99$, $R = 0.992$, 0.990 , and 0.991).

3.2. Size Variation. The size variation study was performed on a total of 590 adult insects (305 females and 285 males). All analyses were carried out separately for each sex. The numbers of wings (n), mean, and standard deviation of CS for each group classified by host and by sex are detailed in Table 1.

The CS of parental wings of either sex was larger than those of their offspring (Table 1), although metric differences were statistically significant only for offspring fed on cockroaches (Table 2). Differences were statistically significant between contemporaneous offspring feeding on different hosts, in females and males from O2 to O3 (Table 2). Wing CS differences between noncontemporaneous offspring fed on the same host were not found, except between males (O1-O2) fed on mice (Table 2).

Kruskal-Wallis comparison between females and males did not show statistically significant differences in SSD among parents ($p = 0.63$), O1 fed on cockroaches ($p = 0.75$), and O1 ($p = 0.0076$) and O3 ($p = 0.036$) fed on mice. However, significant SSD was observed in the O2 ($p = 0.000044$) and O3 ($p = 0.0009$) fed on cockroaches and in the O2 ($p = 0.00046$) fed on mice (Figure 2).

3.3. Allometry. The relationship between wing shape variables and CS showed a significant but low allometric residue: 7.4% in females ($p < 0.001$) and 2.9% in males ($p = 0.0006$).

3.4. Shape Variation. The NPMANOVA of the shape variables (Procrustes coordinates) between parental and female ($F = 19.01$; $p = 0.0001$) or male ($F = 16.55$; $p = 0.0001$) offspring rejected the null hypothesis that the means do not differ. The canonical analysis of the variance in females demonstrated that the first canonical function explained 88% of the total variance and the second function 8%

TABLE 1: The numbers of specimens (n), mean, and standard deviation of centroid size for each group classified by host and sex.

Group	Origin	Sex	Feeding behavior	O	n	Centroid size			
						Mean	Min	Max	SD
P	Field	♀		P	18	1455.84	1361.90	1607.30	67.04
HLO1	Laboratory	♀	Hemolymphagy	O1	45	1341.65	1219.31	1461.48	56.20
HLO2	Laboratory	♀	Hemolymphagy	O2	67	1356.23	1132.53	1481.38	62.30
HLO3	Laboratory	♀	Hemolymphagy	O3	69	1330.59	1230.69	1474.11	45.43
HMO1	Laboratory	♀	Hematophagy	O1	29	1394.37	1297.15	1496.69	52.31
HMO2	Laboratory	♀	Hematophagy	O2	57	1417.21	1228.20	1510.00	57.24
HMO3	Laboratory	♀	Hematophagy	O3	20	1404.68	1238.31	1493.80	58.00
P	Field	♂		P	14	1439.57	1360.48	1512.14	42.59
HLO1	Laboratory	♂	Hemolymphagy	O1	38	1337.52	1154.50	1467.12	62.59
HLO2	Laboratory	♂	Hemolymphagy	O2	71	1314.32	1191.50	1433.39	53.94
HLO3	Laboratory	♂	Hemolymphagy	O3	65	1299.48	1119.23	1403.64	55.65
HMO1	Laboratory	♂	Hematophagy	O1	44	1361.18	1223.34	1476.33	49.83
HMO2	Laboratory	♂	Hematophagy	O2	36	1378.53	1250.72	1461.85	46.44
HMO3	Laboratory	♂	Hematophagy	O3	17	1367.64	1249.47	1469.83	60.27

P (parental), O (offspring), ♀ (female), ♂ (male), O1 (first offspring), O2 (second Offspring), O3 (third offspring), HL (hemolymphagy = fed on cockroaches), HM (hematophagy = fed on mice), n (number of specimens), Min (minimum), Max (maximum), and SD (standard deviation).

TABLE 2: Comparison of centroid size of *B. ferroae*, between parental and progeny reared under laboratory conditions with two different hosts: cockroaches and mice (Kruskal-Wallis test).

Comparison	Sex	
	Females	Males
Parental versus offspring		
P-HLO1	$7.5E - 07^*$	$1.81E - 04^*$
P-HLO2	$1.30E - 05^*$	$8.17E - 09^*$
P-HLO3	$3.40E - 10^*$	$9.7E - 11^*$
P-HMO1	0.3149	0.0201
P-HMO2	1.0000	0.3868
P-HMO3	1.0000	0.1153
Contemporaneous offspring (reared on different host)		
HLO1-HMO1	0.01481	1.000000
HLO2-HMO2	$4.7E - 06^*$	$2.22E - 06^*$
HLO3-HMO3	0.00002*	0.00301*
Offspring noncontemporaneous (reared on the same host)		
HLO1-HLO2	1.000000	0.607057
HLO1-HLO3	1.000000	0.032183
HLO2-HLO3	0.154945	1.000000
HMO1-HMO2	1.000000	0.000435*
HMO1-HMO3	1.000000	1.000000
HMO2-HMO3	1.000000	1.000000

P (parental), HL (hemolymphagy = fed on cockroaches), HM (hematophagy = fed on mice), O1 (first offspring), O2 (second offspring), O3 (third offspring), and * statistically significant difference, $p < 0.0033$.

(Figure 3(a)). In males the first canonical function explained 84% and the second 8% (Figure 3(b)). The Mahalanobis distances between wing shapes of male offspring fed on cockroaches and their parents did not show significant statistical differences. However, female offspring did do so from O2 to O3. All the distances between the wing shapes of all offspring fed on mice and their respective parents were statistically significant (Table 3).

The Mahalanobis distances in wing shape were statistically significant between all offspring feeding on different hosts (Table 3), while differences in wing shape between offspring fed on the same host did not occur (Table 3).

In female offspring, the MANCOVA showed significant differences between slopes (Pillai's trace 0.239; $F = 1.717$; df1 40, df2 1.360; $p = 0.004$), while in males no such differences were observed (Pillai's trace 0.184; $F = 1.221$; df1 40, df2

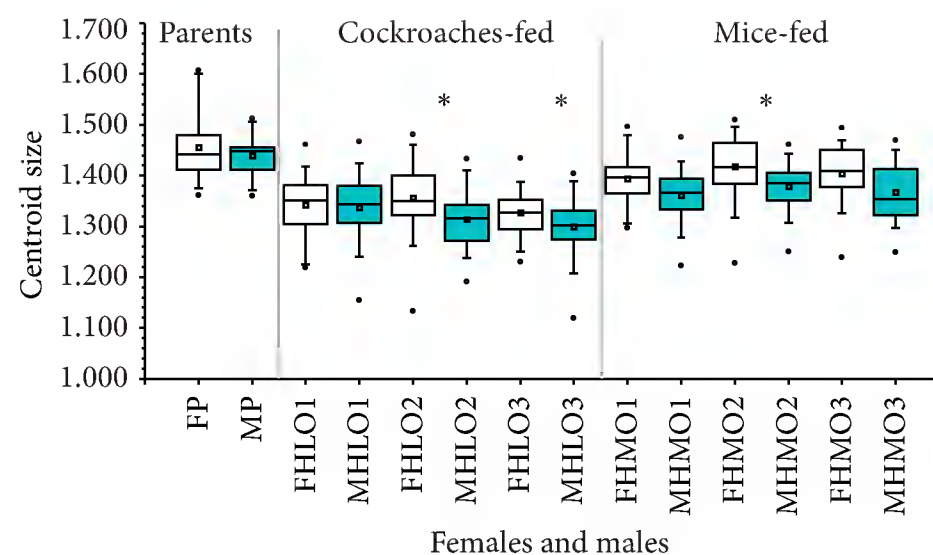


FIGURE 2: Sexual dimorphism in centroid size. FP (♀ parent), MP (♂ parent), FHLO1 (♀O1, hemolymphagy), MHLO1 (♂O1, hemolymphagy), FHMO1 (♀O1, hematophagy), MHMO1 (♂O1, hematophagy), FHLO2 (♀O2, hemolymphagy), MHLO2 (♂O2, hemolymphagy), FHMO2 (♀O2, hematophagy), MHMO2 (♂O2, hematophagy), FHLO3 (♀O3, hemolymphagy), MHLO3 (♂O3, hemolymphagy), FHMO3 (♀O3, hematophagy), MHMO3 (♂O3, hematophagy), O1 (first offspring), O2 (second offspring), and O3 (third offspring). White box (female) and blue box (male). * Statistically significant difference.

1.280; $p = 0.164$). This result showed that size correction could be calculated only for males. The NPMANOVA of the size-corrected shape variables (residuals) between male offspring ($F = 4.52$; $p = 0.0001$) rejects the null hypothesis that the means do not differ between groups. When these residuals were subjected to the CVA, the first canonical function explained 89% of the total variance and the second function explained 5% (Figure 3(c)). All the Mahalanobis distances between male offspring fed on different hosts were statistically significant (Table 3).

3.5. Visualizing Shape Variation. Figures 4 and 5 illustrate the relative changes in wing shape of males and females. The reference or starting configuration in this particular case (sky blue) describes the parental configuration, while the dark blue shows the deformation produced in the parental configuration by superimposing on it the configuration of the offspring. The compared pairs refer to the same ones used in the DA (Table 3). This graph highlights that the differences between the parental configurations and those generations fed on cockroaches are slight. Shape changes' tendencies were similar in females and males of each group raised with a certain host. The changes were located mainly in landmarks 2 and 4 when comparing parents and offspring fed on cockroaches, whereas in the case of insects fed on mice, the relative displacements were located in landmarks 1, 2, 3, and 4 (Figures 4 and 5).

Deformations to the parental configuration of female offspring fed on cockroaches were more pronounced than in males fed on the same host (Figures 4 and 5). The opposite occurred in the insects fed on mice. This graphic result can be verified by means of percentages of reclassification and crosschecking provided by the DA (Table 3).

4. Discussion

4.1. Size Variation. There was an obvious tendency towards wing reduction in both males and females of *B. ferroae* raised with either host (cockroaches or rodents). Nonetheless

such reduction was only significant in those insects fed on cockroaches (Figure 2, Table 2).

For adaptations reasons, it would be reasonable to expect greater morphological stability through similarity in size in individuals fed on cockroaches. However, since *B. ferroae* fed on cockroaches reduced wing size from parents to their offspring, an alternative explanation must be considered.

It is common to find that among *Rhodnius*, *Triatoma*, and *Panstrongylus* genera body size decreases when individuals are laboratory reared for several generations or their habitat changes from sylvan to domestic [14, 21–24]. As far as diet is concerned, wing size variation under natural conditions has been related to the nutritional value of vertebrate hosts [25, 26]. Based on the analysis of the contents of their intestines, *B. ferroae* captured in houses seem to prefer cockroaches as their principal host [4]. This finding is consistent with some demographic analyses where fitness was assessed [2]. But the possibility of a different nutritional value of the invertebrate hosts of *B. ferroae* found in the field and those offered in the laboratory could help to explain the contrasts found in size between parents and offspring fed on cockroaches. In this study, *B. ferroae* was collected together with blatids in rural domiciles where *Periplaneta americana* were present. However, in our study another cooccurring cockroach species (genus *Blaberus*) was used [4].

The experimental design used in this study included differences in host-feeding frequency, daily for the insects fed cockroaches and weekly for those fed on mice. Therefore, heterogeneity in host availability might explain the differences in size, supposing a higher consumption of food in the insects reared on cockroaches. However, one would suppose that bugs fed on mice ingested more food, based on better performance of some component of fecundity such as rhythm (total eggs/female/week during the reproductive period) and intermittence of oviposture (average time between two successive oviposture), in contrast to insects fed on cockroaches, that is, although the last have greater access to the host [2]. For these reasons, one might consider that other dietary factors like host preference must affect growth of the bugs.

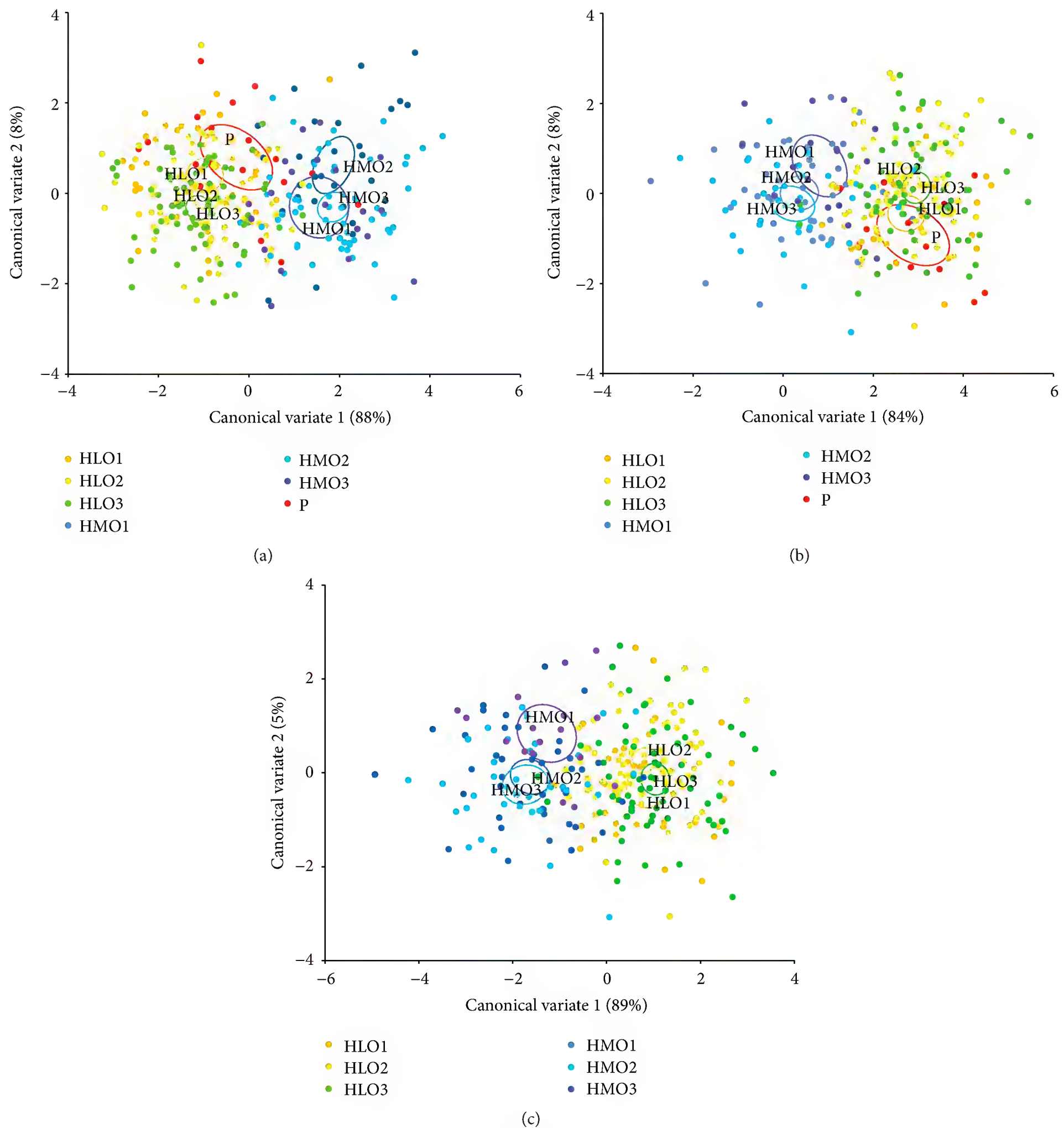


FIGURE 3: Factorial maps in the plane of the two canonical factors of shape variation for wing (CV1 and CV2) presenting the distribution of the phenotypes of wing shape in females (a), males (b), and (c) males offspring (after size correction). The ellipses represent the confidence interval of the average shape of each group with a probability of 95%. P (parent), HL (hemolymphagy = fed on cockroaches), HM (hematophagy = fed on mice), O1 (first offspring), O2 (second offspring), and O3 (third offspring).

Guarneri et al. [27] suggest that the life cycles of *T. infestans* and *T. brasiliensis* bugs were shorter in those fed on mice than in those fed on bird, probably due to their natural history with the mammal. In our study some key aspects related to blood diet adaptation, such as antihemostatic factors, digestive enzyme efficiency [28], evolution of mechanisms to confront the effects of oxidative stress [29], and the adequate

contribution of nutritional factors like vitamin B [30], could be involved in the delayed development time of bugs fed on mice and consequently in the frequency of larger sized individuals in this group.

Size differences among the contemporaneous offspring of *B. ferroae* fed on cockroaches or mice might be explained by the “growth hypothesis” [21], which proposes that long life

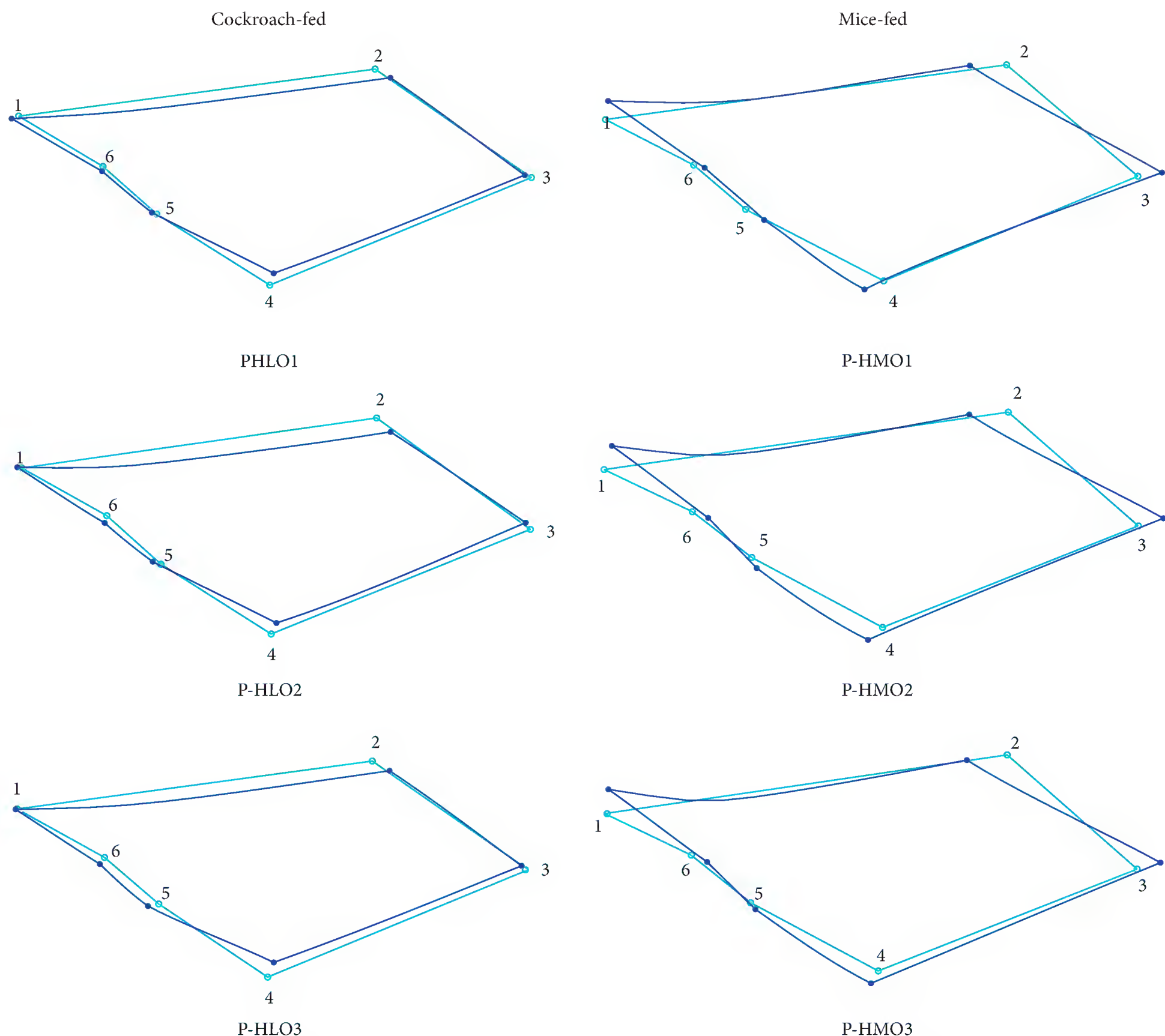


FIGURE 4: Wing deformation caused by superimposing the offspring shape on the parental shape. The sky blue configuration refers to the average shape of the parental females and the dark blue one to the deformation of the parental shape introduced by the laboratory-bred females. Points 1 through 6 refer to the landmarks used to define the wing configuration. (P) Parental, HL (hemolymphagy = fed on cockroaches), HM (hematophagy = fed on mice), O1 (first offspring), O2 (second offspring), and O3 (third offspring).

spans generate large insects and short spans result in small insects. In accordance with this, Nijhout [31] points out that the control of body size is not so much a control of growth but a control of when to stop growing. Final body size is fixed principally by the time available for growth [25, 32]. Some results of the life cycle of *B. ferroae* support this hypothesis, as development time from nymph to adult was significantly shorter ($p < 0.001$) in the insects fed on cockroaches (135.2–144.2 days) than in those fed on mice (187.0–203.5 days) [2].

Finally, it is important to point out that our experimental conditions were not the same as those that the insects find upon infesting human dwellings: availability of hosts, diversity of hosts offered (arthropods and vertebrates), and other

factors, for example, photoperiod, temperature, and relative humidity, all likely influenced the size of the insects [23, 25]. In general, the principal factors that affect body size in insects under laboratory conditions are temperature, crowding, endogamy, diet (nutrient quality), and host [10, 14, 25, 33–35]. As temperature and crowding were constant in our current study, size differences among contemporaneous offspring fed on cockroaches or mice cannot be ascribed to variations in these factors. Similarly, the endogamy does not seem to have an important influence because no significant size differences were found between noncontemporaneous offspring reared with either host. Our results showed that size was stabilized from the first generation on similar to observations found in *P. geniculatus* [23].

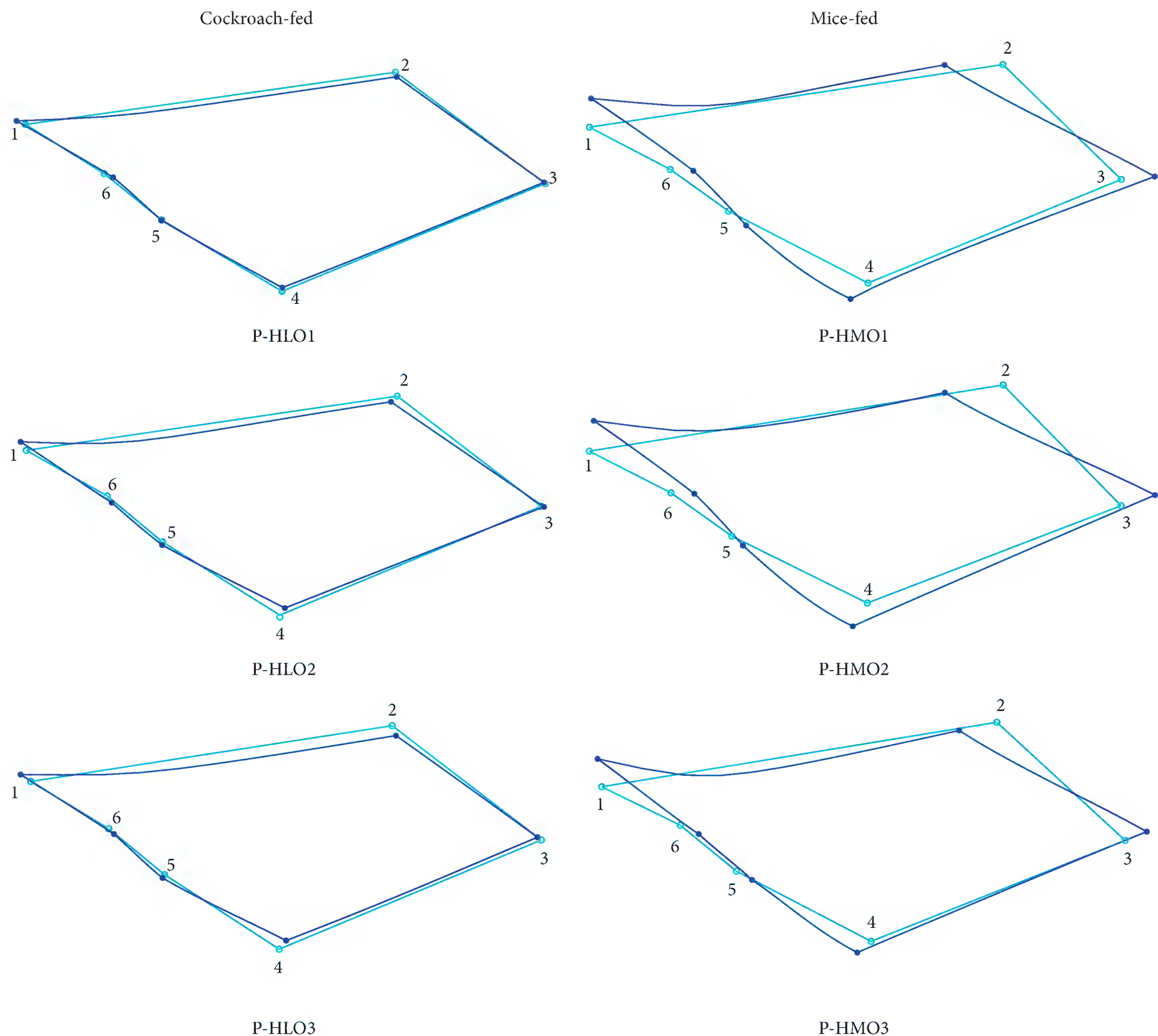


FIGURE 5: Wing deformation caused by superimposing the offspring shape on the parental shape. The sky blue configuration refers to the average shape of the parental males and the dark blue one to the deformation of the parental shape introduced by the laboratory-bred males. Points 1 through 6 refer to the landmark used to define the wing configuration. (P) Parental, HL (hemolymphagy = fed on cockroaches), HM (hematophagy = fed on mice), O1 (first offspring), O2 (second offspring), and O3 (third offspring).

Sexual size dimorphism (SSD) in Triatominae is well known as females are generally larger than males [36]. Nijhout [31] points out that large individuals store larger and better resources than the smaller ones. A large body could be more useful to a female than to a male because it increases the probability of survival and production of a greater number of eggs [37, 38]. SSD is frequently considered adaptive in reproduction, as larger individuals would have a greater opportunity to be chosen by their mate [38].

However, within a species, the degree of SSD may be affected by environmental conditions [39]. Quantitative analysis of changes in size of species like *R. domesticus*, *R. prolixus*, and *T. infestans* suggests that reduction in the average size of females may lead to diminished sexual dimorphism [22] as a

result of more stable conditions found in human dwellings or laboratories. Thus, reduction in this morphological character has been proposed as a marker of domiciliation in Triatominae [22–24]. The fact that SSD persists in *P. geniculatus* after five laboratory generations suggests that more generations are necessary for this phenomenon to be manifested [23].

The absence of SSD in *B. ferroae* collected in human dwellings could be interpreted as evidence that adaptation to this environment is not recent and that more than five generations had been raised there. However, the presence in this study of SSD in offspring of *B. ferroae* fed on either cockroaches or mice shows that under stable breeding conditions SSD certainly may occur. Therefore, in spite of the consistent size reduction of the offspring of *B. ferroae*, it is

TABLE 3: Comparison of wing shape of *B. ferroae*, between parental and offspring reared under laboratory conditions with two hosts (cockroaches and mice). Mahalanobis distances (Md) and their significance (p) obtained from CVA and classifications and cross-check from DA.

Comparison	Females						Males					
	Procrustes coordinates			Residuals (after size correction)			Procrustes coordinates			Residuals (after size correction)		
	Md	p	% Classif.	% Cross-check	Md	p	% Classif.	% Cross-check	Md	p	% Classif.	% Cross-check
Parental versus offspring												
P-HLO1	1.0127	0.175	77-66	50-60	0.8561	0.4955	71-63	43-58				
P-HLO2	1.3408	0.003	72-81	55-76	1.2671	0.0103	71-73	50-70				
P-HLO3	1.5354	<0.0001*	72-80	66-78	1.3038	0.0389	79-74	50-68				
P-HMO1	2.338	<0.0001*	89-96	78-86	2.7723	<0.0001*	100-93	79-90				
P-HMO2	2.4613	<0.0001*	78-91	72-82	2.8637	<0.0001*	86-92	64-81				
P-HMO3	2.2396	<0.0001*	83-95	66-70	2.658	<0.0001*	79-100	64-88				
Offspring (reared on different host)												
HLO1-HMO1	2.8991	<0.0001*	93-100	82-97	2.4688	<0.0001*	90-90	87-83	2.5807	<0.0001*	55-64	55-64
HLO1-HMO2	2.9373	<0.0001*	91-91	87-89	2.6063	<0.0001*	92-92	84-83	2.7678	<0.0001*	66-67	66-67
HLO1-HMO3	2.662	<0.0001*	89-95	84-90	2.2699	<0.0001*	90-94	87-88	2.4968	<0.0001*	55-53	55-53
HLO2-HMO1	2.9739	<0.0001*	96-89	91-86	2.4497	<0.0001*	92-91	87-90	2.612	<0.0001*	70-73	69-73
HLO2-HMO2	2.8772	<0.0001*	90-88	89-86	2.6409	<0.0001*	93-89	92-86	2.8634	<0.0001*	77-75	77-75
HLO2-HMO3	2.5492	<0.0001*	89-90	85-80	2.1027	<0.0001*	89-88	85-77	2.4354	<0.0001*	73-65	71-65
HLO3-HMO1	3.1613	<0.0001*	94-97	93-86	2.6471	<0.0001*	94-89	94-84	2.9286	<0.0001*	74-80	74-80
HLO3-HMO2	3.0115	<0.0001*	94-93	94-84	2.7983	<0.0001*	92-89	91-89	3.1581	<0.0001*	83-81	83-81
HLO3-HMO3	2.7788	<0.0001*	97-100	96-90	2.3596	<0.0001*	94-89	94-100	2.8389	<0.0001*	80-65	80-65
Offspring (reared on the same host)												
HLO1-HLO2	0.6264	0.2769	62-64	51-58	0.8409	0.0122	58-70	56-62	0.866	0.0151	66-56	66-55
HLO1-HLO3	0.8455	0.0095	60-64	53-58	0.7487	0.1196	58-63	47-54	0.9359	0.018	66-60	66-60
HLO2-HLO3	0.7086	0.015	69-67	66-65	0.5363	0.2792	61-64	55-52	0.6454	0.1183	55-52	55-51
HMO1-HMO2	1.0607	0.0099	72-72	58-64	0.7865	0.1514	75-64	59-50	0.8317	0.1515	52-58	52-58
HMO1-HMO3	1.2678	0.0416	69-80	62-60	1.1701	0.0289	80-71	80-53	1.2126	0.0403	50-41	50-35
HMO2-HMO3	0.6175	0.6581	71-60	57-35	1.3165	0.0095	73-77	61-71	1.3128	0.0196	56-65	56-65

P (parent), HL (hemolymphagy = fed on cockroaches), HM (hematophagy = fed on mice), O1 (first offspring), O2 (second offspring), O3 (third offspring), % Classif. (% classification), and * statistically significant difference, $p < 0.0024$.

difficult to support the hypothesis of reduced dimorphism proposed by Dujardin et al. [22] in insects under stable breeding conditions as in a laboratory. This result differs from previous observations made in other groups of insects in which the increase in the degree of SSD is generally accompanied by an increase in the body size of females [39]. In our study of *B. ferroae*, the female's wing size, which might be considered as a global estimation of body size, did not vary significantly from one generation to the next (see Table 2) in spite of the appearance of sexual dimorphism. Therefore it is reasonable to suppose that other factors, different from those traditionally used to explain the presence or degree of SSD, could be operating in this species, as has been proposed [38] in Trichoptera. These results demonstrate the need for a cautious interpretation of changes in SSD of laboratory offspring as an analog for domiciliation in Triatominae.

4.2. Shape Variation and Allometry. The choice of a host affects morphophysiological aspects of the Triatominae [9, 40], as well as reproductive and demographic parameters that determine fitness, including development times [2, 27], mortality, and fecundity rates [41–44].

Recently, the host (pigeon and guinea pig) was demonstrated to influence head shape in *T. infestans* [11]. In general terms, shape shows greater stability than size under the influence of certain environmental factors [45]. For example, in the triatomine *R. pallens*, density and host availability modified the size of wings and heads, but not their shape [14].

In *B. ferroae*, wing data revealed that size contributed significantly to variation of shape (allometric effect). Thus, differences in shape cannot be attributed principally to this effect because the contribution, although significant, is small here and in other species [10, 11, 46]. If an important percentage of the variation of shape is independent of size, then other factors must be involved in the differences of wing shape between groups. In this study, insects were derived from a single population, and environmental factors such as temperature, relative humidity, photoperiod, and density were controlled. Therefore, it is possible that differences in shape are related to the host. In this sense, the results obtained in the analysis of wing shape (free allometry) of male offspring are interesting, where significant differences between the shapes of the generations fed on different hosts (cockroaches versus rodents) appear. This observation confirms that the previously observed shape differences were not simply allometry and supports the hypothesis of host effect on wing morphology (Table 3).

Several studies have shown the influence of host or diet on the induction of morphological changes. Andersson et al. [47] showed that in the carp *Carassius carassius* changes in body shape were induced by diet (zooplankton or benthic chironomids). In *Drosophila (repleta)* group, shape of the aedeagus changes depending on the cactus host [48].

Here, in *B. ferroae*, the canonical analysis of the variance showed that the Mahalanobis distances were always greater and significant between the phenotype of parental wing shape and that of descendants fed on mice, whether female (2.239–2.461) or male (2.658–2.863). On the contrary, the Mahalanobis distances between the parental phenotype and

that of the descendant insects fed on cockroaches were always lesser in the females (1.0127–1.5354), with significant differences only after the third generation and in all the males (0.8661–1.3038), without any statistically significant differences among them for the three laboratory generations. These results indicate a greater similarity in wing shape between parents and offspring raised with cockroaches.

Our results imply that the parental population had fed on invertebrate hosts similar to those used in here and support the viability of the life cycle of *B. ferroae* with cockroaches in natural conditions (human dwellings). These data, coupled with the information relative to the alimentary profile of *B. ferroae* captured in houses that showed that 89.6% of the intestinal contents had hemolymph, probably from cockroaches [4], and with the life history analysis that showed a greater demographic fitness with cockroaches [2], constitute ecological, demographic, and morphological evidence of adaptation of this species to invertebrate hosts.

In addition, the superimposition of the wing shape of females fed on cockroaches produced a greater degree of deformation to the parental configuration than that of males (see Figure 4). As a cause to such deformation, we propose that in the field females may be more eclectic in their diet than males due to different nutritional requirements of the sexes. The simultaneous detection of hemolymph and mammal blood (human and rodent), in the intestinal contents of *B. ferroae* captured in human dwellings, supports this possibility [4].

Furthermore, although the association of *B. ferroae* with vertebrate hosts does not appear to be common under natural conditions (dwellings) and in laboratory conditions showed a low fitness [2], this association could be vital on those occasions where the arthropod host is unavailable. Additionally, feeding on mammal hosts may have relevant epidemiological consequences as it would contribute to the preservation of *T. cruzi*'s cycle in nature, given the susceptibility of *B. ferroae* to infection by this parasite [49].

In *B. ferroae* the wing shapes exhibited by insects fed on mice could be interpreted as the consequence of phenotypic plasticity in insects submitted to stressful conditions, considering the minimally adaptive value (low population growth rate) of such a diet [2].

In conclusion, diverse tendencies demonstrated by the form components, size and shape, suggest that the observed differences may be due to a relative independence of these two aspects of wing morphology [10]. It seems likely that variations in size and shape are influenced by different external variables. For example, size could be more sensitive to small variations in the availability and utilization of resources [50].

Our analysis of phenotypical variation was facilitated by geometric morphometry techniques and made it possible to detect changes in shape produced by a pair of potential hosts (cockroaches and mice) in *B. ferroae*. In addition, the high similarity in wing shape found between parental and offspring fed on cockroaches is consistent with the idea that *B. ferroae* is not an obligate hematophage [2] and that in its spectrum of hosts it prefers arthropods and only occasionally mammals, birds, or reptiles [4].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Temporal and Spatial Foraging Behavior of the Larvae of the Fall Webworm *Hyphantria cunea*

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During their first three larval stadia, caterpillars of *Hyphantria cunea* (Lepidoptera: Arctiidae) are patch-restricted foragers, confining their activity to a web-nest they construct in the branches of the host tree. Activity recordings of eight field colonies made over 46 colony-days showed that the later instars become central place foragers, leaving their nests at dusk to feed at distant sites and then returning to their nests in the morning. Colonies maintained in the laboratory showed that same pattern of foraging. In Y-choice laboratory experiments, caterpillars were slow to abandon old, exhausted feeding sites in favor of new food finds. An average of approximately 40% of the caterpillars in five colonies still selected pathways leading to exhausted sites at the onset of foraging bouts over those leading to new sites after feeding exclusively at the new sites on each of the previous four days. On returning to their nests in the morning, approximately 23% of the caterpillars erred by selecting pathways that led them away from the nest rather than toward it and showed no improvement over the course of the study. The results of these Y-choice studies indicate that, compared to other previously studied species of social caterpillars, the webworm employs a relatively simple system of collective foraging.

1. Introduction

While sibling aggregates of caterpillars are simple societies lacking the complexity of interaction that characterizes the classically eusocial insects, studies show that the various species exhibit a rich diversity of cooperative interactions. Indeed, collectively, social caterpillars exhibit a greater range of social behaviors than are found among those species traditionally classified as subsocial forms and eclipse those of species of thrips, beetles, and aphids recently added to the catalog of eusocial insects [1, 2]. Their social repertoires include communal and cooperative foraging, trail based communication and recruitment, cooperative shelter building, collective thermoregulation, and antipredator defense [2].

Much of the interest in social caterpillars has focused on their collective foraging behavior. The most common mode of feeding found among social caterpillars involves patch-restricted foraging [2, 3]. Species exhibiting this mode of foraging envelop patches of leaves with silk and feed within the web-nest. As they grow, they may enlarge their feeding

arenas to include a series of more or less contiguous patches often linked by silk covered branch pathways. One of the most widely distributed and conspicuous patch-restricted foragers is the fall webworm, *Hyphantria cunea* (Lepidoptera: Arctiidae). In the northeastern USA, the moths appear in the late spring and oviposit several hundred eggs on the undersides of the leaves of host plants [4]. The caterpillars eclose from their eggs in late June and immediately initiate the construction of a web-nest which they inhabit for all of their larval life. As the caterpillars grow, they continually extend the boundaries of the nest to enclose new leaves on which they feed.

Berger [5] and Snodgrass [6] were the first to publish observations of the temporospatial foraging behavior of the fall webworm. While both agreed that the caterpillars are initially patch-restricted foragers, Berger [5] stated that, during the later stadia, caterpillars in colonies he observed travelled to distant feeding sites outside the nest to feed at night, thus behaving as central place foragers. Snodgrass [6], however, questioned these observations, stating that the colonies he observed fed solely within the nest throughout



FIGURE 1: Infrared activity monitor.

their larval life. The distinction is significant because central place foraging sets the stage for the evolution of more advanced forms of cooperative foraging than occur among those species that are strictly patch-restricted foragers [3]. While other investigators have since reported on the temporal and spatial pattern of the foraging behavior of the webworm [7–9] neither these studies nor those of Berger [5] and Snodgrass [6] provide hard documentation of the temporal and spatial pattern of foraging of undisturbed field colonies. The present study was undertaken to provide that data set. The efficiency with which the colonies abandon exhausted feeding sites in favor of new food finds and the ability of caterpillars to find the way back to the nest from distant feeding sites were also investigated.

2. Materials and Methods

2.1. Source of Caterpillars. Studies of the temporal and spatial pattern of foraging of field colonies were conducted in 1997 on caterpillars collected from Cortland County, NY. Laboratory studies to determine the efficiency with which caterpillars abandoned exhausted feeding sites and their ability to return directly to the nest after feeding were conducted in 2014 on caterpillars collected from Lycoming County, PA.

2.2. Temporal and Spatial Pattern of Foraging. In addition to direct observations of field colonies made throughout their development, electronic recording of eight field colonies was undertaken from August 20 to September 21, 1997, to obtain information on the daily pattern of foraging of maturing caterpillars. To record colony activity, an infrared activity monitor was attached to the main branch-pathway extending from the nest in such a manner that caterpillars moving between their nests and feeding sites broke the infrared beam and triggered the detector ([10], Figure 1). Fall webworms spin copious quantities of silk and it was necessary in some instances to remove silk from the monitor at the onset of activity periods to assure that the caterpillars passed between the detector's emitter and sensor. Detector response was registered on a Hobo event logger (Onset Computer Co., Bourne, MA, <http://www.onsetcomp.com/>) and the data later uploaded to a computer and compiled with BoxCar software

(Onset Computer Co.). Activity over a total of 46 colony-days was recorded.

2.3. Efficiency of Abandoning Exhausted Feeding Sites. Y-choice experiments were conducted that required caterpillars to choose between alternate pathways, one of which led to an old, exhausted feeding site and the other to a new site. The nests of fifth to sixth instar caterpillars used in these studies were collected in the field and established on separate wooden stands made of 1/4 inch dowels to resemble the branching pattern of a tree [11]. Colonies readily adapted to the stands, gradually expanding their nests over the expanse of the structures. Caterpillars in each colony were provided with a fully foliated branch of black cherry (*Prunus serotina*) as a food source which was abutted to the end of an elongated horizontal arm of the stand (the bridge). The branches were sufficiently large to provide the caterpillars with leaves in excess of their needs. The colonies were maintained in a room with windows admitting daylight so that the caterpillars were exposed to the same light regime as in the field. Experiments were initiated after the caterpillars had been established on the stands for at least one week. To conduct a Y-choice experiment, two additional sections of dowels, each 20 cm in length and previously unused, were arranged to extend the bridge leading to food. The proximal section of the extension was placed in line with the bridge while the distal section was abutted to the end of the proximal section at an angle of 15 degrees and the cherry branch placed at its end. Caterpillars were allowed to move over these sections and to feed *ad libitum* for the next two days. On the third day, another 20 cm long section of unused dowel was abutted to the end of the proximal section of the extension at an angle of 15 degrees. Thus the proximal stem section and the two distal sections (arms) were arranged in the form of a Y, separated by an angle of approximately 30°. Leaves remaining on the branch that the caterpillars had been feeding on the previous two days, located at the end of the first arm, were removed by hand but the branch was left in place and it and the arm leading to it were otherwise undisturbed. A new, fully foliated branch was placed at the end of the new arm. The number of caterpillars selecting each of the alternative arms of the Y as they moved from their nests to feed was determined by analyzing video recorded at the rate of one frame per second during each of the following five days. Thus, the study was designed to determine how long it would take the caterpillars to abandon the arm that led to the exhausted feeding site in favor of the arm that led to a new food supply. Night-time recording was facilitated by illuminating the Y with a 25 w red light bulb. The study was replicated with five different colonies of fifth to sixth instar caterpillars.

2.4. Ability of the Caterpillars to Return Directly to the Nest after Feeding. As caterpillars return to their nest after feeding they must select between alternate pathways at branch junctures. The extent to which caterpillars select pathways that provided the most direct route back to the nest was observed under laboratory conditions. Caterpillars in each of the five laboratory colonies, established as described above, were monitored by camera as they returned to their nests after

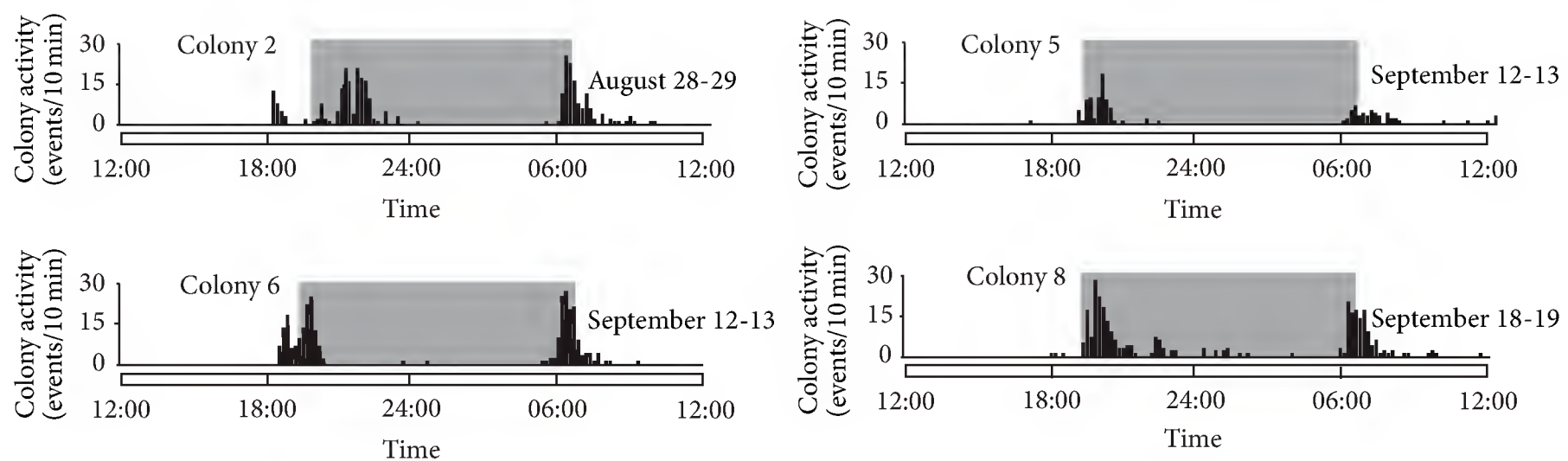


FIGURE 2: Activity records of field colonies of *Hyphantria cunea* caterpillars recorded in 1997. Grey rectangles indicate periods of darkness.

TABLE 1: Activity median (minutes $\pm$ SE) after sunset (evening) and before sunrise (morning) for eight colonies of *Hyphantria cunea*.

Colony	Days recorded	Activity median	
		Evening	Morning
1	5	62.8 $\pm$ 7.6	38.0 $\pm$ 6.9
2	7	67.7 $\pm$ 12.6	7.3 $\pm$ 4.6
3	5	54.2 $\pm$ 16.6	6.0 $\pm$ 9.0
4	3	87.6 $\pm$ 9.6	36.0 $\pm$ 7.5
5	8	34.7 $\pm$ 4.5	1.3 $\pm$ 7.8
6	8	51.4 $\pm$ 7.6	1.0 $\pm$ 7.1
7	8	78.4 $\pm$ 13.2	20.7 $\pm$ 8.6
8	2	57.5 $\pm$ 4.5	0.5 $\pm$ 5.5
All colonies	46	59.7 $\pm$ 4.4	12.7 $\pm$ 3.1

feeding overnight. When a caterpillar reached the juncture of the stem and the alternate arm of the Y, it had to select between the two: the stem leading to the nest or the alternate arm leading away from the nest. Caterpillars choosing the alternate arm and moving along it to the old, exhausted feeding site were considered to make a mistake as this choice eventually required them to turn around to find their way back.

2.5. Statistical Analysis. Data were analyzed with SigmaStat statistical software. Proportions were subjected to arcsin sqrt transformation before analyses.

3. Results

3.1. Temporal and Spatial Pattern of Foraging. Direct observations of field colonies indicate that through the third stadium the caterpillars forage within the nest which is continually expanded to enclose new leaves. Recordings show that, in the subsequent stadia, the caterpillars are central place foragers, leaving the nest at dusk and traveling to distant feeding sites. Activity records of four colonies, typical of the temporal pattern of behavior exhibited by all eight of the field colonies, are shown in Figure 2. The activity median of caterpillars exiting the nest occurred approximately one hour after sunset (Table 1). Caterpillars in field colonies fed overnight then returned to their nests in the morning with the activity

median of returning occurring approximately 13 minutes before sunrise. Although the majority of the caterpillars were back in the nest by 0800 h, stragglers sometimes did not return until later in the morning (Figure 2). Caterpillars in all of our laboratory colonies maintained under an ambient daylight regime showed a similar pattern of nocturnal foraging (Figure 3).

3.2. Efficiency of Abandoning Exhausted Feeding Sites. During the first foray after the food supply was switched from the old to the new site, caterpillars initially selected the arm of the Y that led to the old site. After exploring the defoliated branch the caterpillars returned to the nest and then started out again, often several times. Some of the caterpillars eventually ventured onto the new arm of the Y but initially turned back after moving part way along its length. As they moved onto the new arm the caterpillars laid down strands of silk and the pathway became increasingly covered with the material. Successive waves of caterpillars to move onto the new arm of the Y showed decreased hesitancy and a lessened tendency to turn back. Trail marking in the FWW has not yet been investigated so it is not known if the physical/chemical properties of silk elicit trail following or if trail marking involves a pheromone secreted from some other site as has been shown for other caterpillars [2]. The time elapsed between the onset of the first foray after the food was switched and the arrival of the first caterpillar at the new food source was highly variable among colonies. The median interval was 27 minutes. Thereafter, caterpillars in increasing numbers selected the arm of the Y leading to the new food source and eventually all of the colony moved to the new site and fed. Of the total number of choices between Y arms made during the first foray after the feeding site was moved, $33.1 \pm 6.3\%$ were for the new site. On the following evening $44.7 \pm 6.9\%$ were for the new site and on the third evening $58.9 \pm 5.4\%$ were for the new site (Figure 4). In all cases, all caterpillars in each colony eventually found their way to the new feeding site. Although the proportion of choices for the new site increased between the first and third days, it did not increase on the two subsequent days on which there was a marked variation in response among colonies (Figure 4). For the overall data set, there was no significant difference in the number of choices for the new site over time (ANOVA, $F = 1.75$, $p = 0.18$).

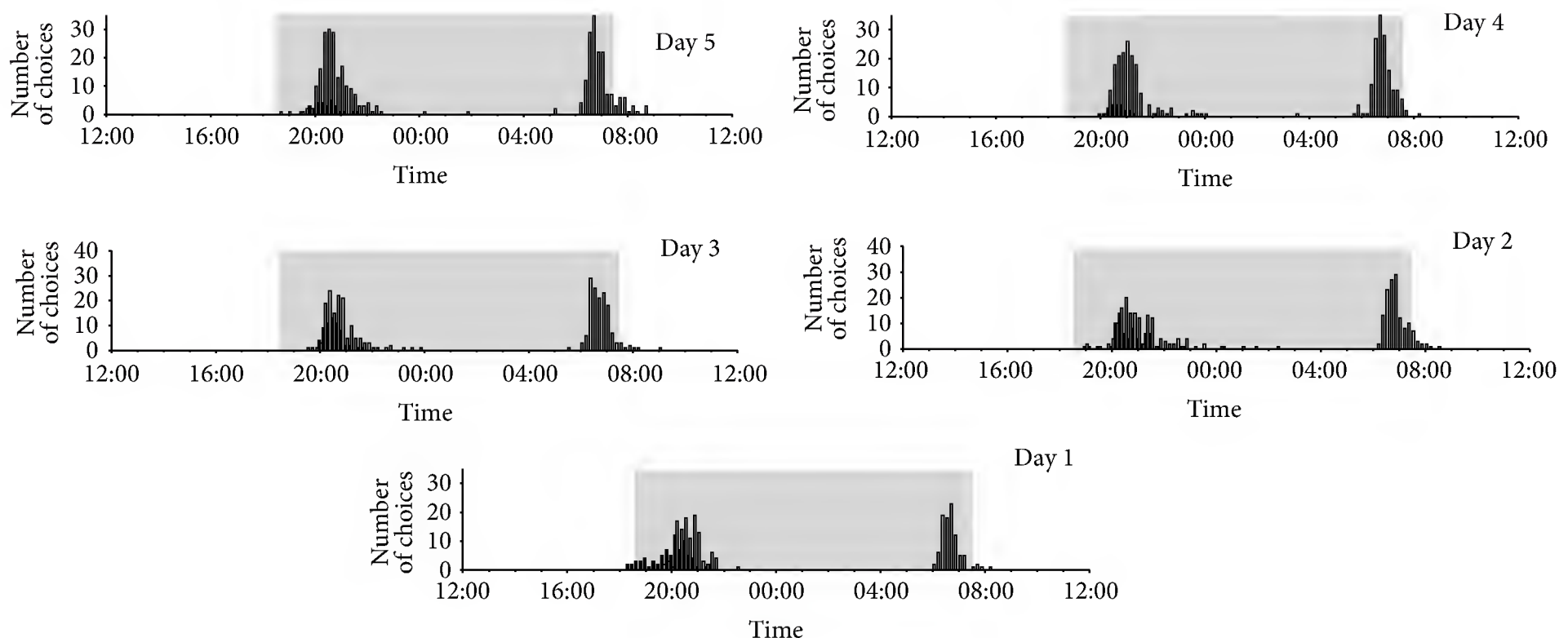


FIGURE 3: Activity patterns of caterpillars in a laboratory colony recorded over five consecutive days. The x-axis records the number of choices over five-minute intervals for the old site (dark bars) and the new site (hollow bars). Scotophase indicated by shaded area.

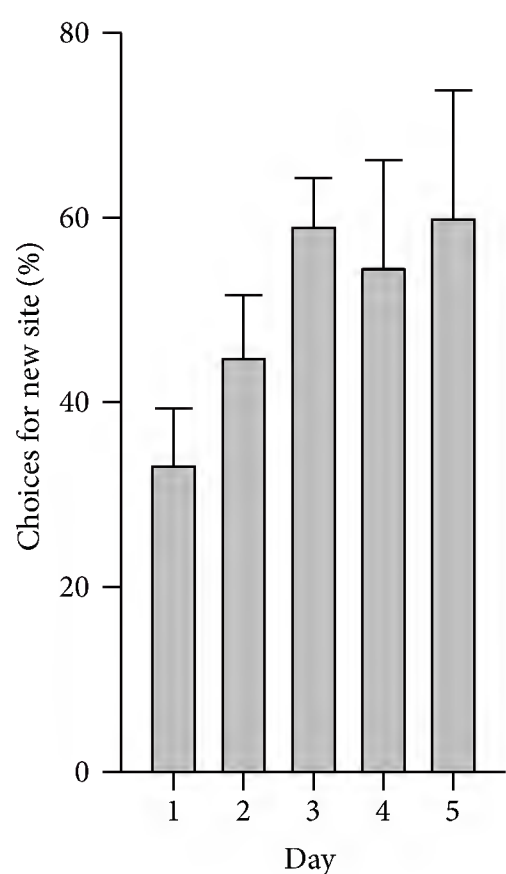


FIGURE 4: Mean percent ($\pm$ SE) choices by fall webworm caterpillars for arms of Ys leading to new feeding sites at onset of evening foraging bouts.

3.3. Ability of the Caterpillars to Return Directly to the Nest after Feeding. On returning to the nest on the first morning after the food supply was switched from the old to the new site $22.5 \pm 8.8\%$ of the caterpillars in the five colonies selected and moved onto the arm of the Y that led to the old feeding site rather than the stem of the Y that led directly to the nest. On the second and third days of the experiment, $21.2 \pm 7.8\%$ and $25.4 \pm 7.1\%$ of caterpillars, respectively, initially chose the arm that led to the old feeding site over the stem. There was no difference among responses over the three days (ANOVA, $F = 0.09$, $p = 0.92$).

4. Discussion

4.1. Temporal and Spatial Pattern of Foraging. In this study, late instar caterpillars adopted a central place foraging mode consistent with the observations first reported by Berger [5]. Although Snodgrass [6] disagreed with Berger on this point, stating that the colonies he observed foraged only in the proximity of the nest, subsequent studies suggest that Snodgrass observed a population of the caterpillar that behaved differently than the caterpillars observed by Berger. There are two color morphs of the FWW and Oliver [7] noted that while the larvae of both morphs he studied in Arkansas fed in contact with the nest during the early instars, during the last three stadia, caterpillars of what he termed the “orange race” left the nest at night to feed and then returned in the morning, whereas those of the “black race” did not. Ito and Warren [8] and Takeda [9] also reported that the behavior of the two color types differed in a manner consistent with that first noted by Oliver [7] though they referred to the lighter colored caterpillars as a “red headed type.” There is no reproductive isolation between the color forms and Morris [12] and Hattori and Ito [13] observed colonies with “mosaic” head colors that behaved like the red headed type. A distribution map drawn by Ito and Hattori [14] shows the black headed form occurring in a region that includes Connecticut where Snodgrass [6] made his observations and a mosaic form occurring in Ohio where Berger [5] made his. Indeed, Snodgrass described his caterpillars as having black heads. Berger, however, reported considerable variation in the head colors of the caterpillars in the colonies he studied, ranging from black to light brown, but did not record any differences in the temporal or spatial foraging patterns of the colonies; all adopted the central place mode of foraging.

Although Ito and Hattori [14] concluded that the red headed type was restricted to the southern United States, both the red and black headed forms were common in Cortland

County, New York, in 1997 when the first phase of the study reported here was conducted. Of 18 colonies observed in the study area, seven consisted of purely black headed caterpillars, two of purely red headed caterpillars, and eight of a mixture of both black headed and red headed caterpillars. In 2014, colonies were collected from near Williamsport, Pennsylvania, as no local colonies could be found. All of 20 colonies that were collected in Pennsylvania consisted of caterpillars with the mosaic head color. As reported here, all caterpillars in the eight colonies recorded in 1997 and the five colonies in 2014 foraged only at night at sites distant from the nest and then returned in the morning. The colonies whose behavior was recorded in 1997 were of mixed black and red headed types with the exception of colony 4 which was a colony of the pure black headed type. As shown in Table 1, colony 4 exhibited the same pattern of nocturnal foraging as the mixed colonies. The results of this and the previous studies noted above indicate that there is considerable temporal and spatial heterogeneity in the relationship between color morphism and larval foraging patterns in the webworm.

4.2. Ability of the Caterpillars to Return Directly to the Nest after Feeding. Caterpillars observed in field colonies during the present study broke into small groups or fed alone when they left the nest in the evening and became widely dispersed, yet all the caterpillars eventually returned to the nest each morning. Observations made while caterpillars were returning to their nests following these overnight forays both in the laboratory and in the field indicate that the webworm extracts less information from its pathways than previously studied species of central place foragers [2]. Studies of these other species showed that they are able to distinguish between pathways at branch junctures that differ in strength and age and that this allows caterpillars to move efficiently from the feeding site back to the nest. Unlike these other species, between 22 and 25% of the choices made by webworms in our laboratory colonies at the Y path juncture on three consecutive mornings as they returned to the nest after feeding overnight were for pathways that led away from the nest rather than toward it. Caterpillars returning to their nests following their overnight feeding bouts in the field were also observed to make mistakes at branch junctures that took them farther away from their nests. During one set of observations, each of five-minute duration, made as caterpillars were returning to their nests in the morning, an average of 9.7 ± 2.2 caterpillars ($n = 3$ colonies) were observed to make choice errors that resulted in them moving away from rather than toward their nests. In addition, as shown in Figure 2, a small percentage of caterpillars in colonies typically returned to their nests much later in the morning than the majority suggesting that these individuals could have had particular difficulty finding their way back.

4.3. Efficiency of Abandoning Exhausted Feeding Sites. Although there have been no studies of chemical communication in the larval webworm, the results of the study presented here indicate that, in contrast to other social caterpillars

that are central place foragers, the webworm employs a relatively simple system of communication that may do a little more than facilitate aggregation and collective nest construction. Compared to the eastern tent caterpillar, an ecological equivalent of the webworm which feeds on *P. serotina* in the spring and is a central place forager, the present study shows that the webworm is relatively inefficient not only in finding its way back to its nest but also in abandoning exhausted feeding sites in favor of new food finds. When tested under similar conditions using the same experimental protocol, colonies of the tent caterpillar abandoned exhausted feeding sites on the same evenings that they discovered and fed at new sites and did not return to the old sites on subsequent evenings [15]. In contrast, a significant proportion of the caterpillars in our laboratory colonies of webworms continued to return to exhausted feeding sites even after feeding for the previous four days at new food finds. This rapid shift to a new feeding site by the tent caterpillar is attributable to the ability of the insect to recruit nestmates to new food finds with a trail pheromone [15]. The central place foragers *Eucheria socialis* [16], *Gloveria* sp. [11], and *Eutachyptera psidii* [17] tested in the same manner showed that while they are less efficient than the tent caterpillar in abandoning depleted sites, they performed markedly better than the webworm with fewer than 20% of the caterpillars returning to the old site on the second night following the shift in the food source. In contrast, a similar study of the pine processionary, *Thaumetopoea pityocampa* [15], showed that the caterpillars of this species are slow to move to a new feeding site with approximately the same percentage of caterpillars as the webworm choosing the arm that led to the old depleted feeding site on the third day of the study.

Although fall webworms may show some preference for leaves based on age and extent of shading [18], they are relatively nonselective in their choice of food compared to *M. americanum*, the larvae of which strongly prefer the youngest leaves, which occur at the tips of branches [19]. In the spring when tent caterpillars are feeding on *P. serotina* there is high heterogeneity for leaf age as the trees continuously produce new leaves. The same host trees have largely stopped producing new leaves when the larvae of the webworm eclose and for the most part the caterpillars feed on aged leaves throughout their larval life that are tough and dry, for which little or no searching is required. The difference in the amount of information that the webworm and the tent caterpillar encode in and extract from their pathways likely reflects this difference in their response to leaf quality and the patchiness of the food resource.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Pimachrysa (Neuroptera: Chrysopidae: Nothochrysinae): Larval Description and Support for Generic Relationships

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We describe the previously unknown larval characteristics of *Pimachrysa* (second and third instars), and we provide new comparative data on the *Nothochrysa californica* Banks larvae that were described earlier. The *Pimachrysa* larvae (identified as *Pimachrysa fusca* Adams) express the numerous features that characterize the chrysopid subfamily Nothochrysinae. They resemble *Hypochrysa* larvae in many respects, but several differences support retaining the two genera as separate. It now appears that the reputedly more archaic, small bodied, nothochrysine genera (e.g., *Dictyochrysa*, *Hypochrysa*, and *Pimachrysa*) have naked larvae that differ distinctly from the larvae of *Nothochrysa* species, all of which are debris-carriers. In addition, the pattern of variability in *Nothochrysa* larval features supports the proposal that this genus has a closer relationship with the other chrysopid subfamilies than do the small-bodied nothochrysine genera. The findings are consistent with the hypothesis that larval nakedness (as opposed to debris-carrying) was the ancestral life style for the extant chrysopid clade.

1. Introduction

Considerable effort has focused on elucidating the evolutionary history of the neuropteran family Chrysopidae, and yet significant challenges remain. The subfamily Nothochrysinae is of particular interest, largely because of its position at or very near the base of the chrysopid phylogenetic tree and also because of its substantial and ancient fossil record. Members of this subfamily have primitive patterns of wing venation and unique features in their larval morphology that have been used to explore evolutionary trends within the subfamily [1–6]. However, generic relationships among the extant taxa remain ambiguous. On-going molecular studies [4, 7, 8] thus far have yielded some interesting, but contradictory results concerning the origin of the subfamily, and they have not yet resolved the relationships among its constituent genera.

Ideally, the above issues would be met with systematic comparisons among all included taxa using a broad range of

approaches (adult morphology, larval morphology, comparative biology, and molecular sequencing). In reality, a large barrier prevents such an analysis, that is, the rarity of specimens from relevant taxa. For example, except for a few species, specimens of Nothochrysinae and other basal chrysopid taxa are seldom encountered in the field; as a result the range of taxonomic coverage has been relatively narrow.

To continue efforts in overcoming this barrier, we describe the hitherto unknown larva (Semaphoront B) of the nothochrysine genus, *Pimachrysa*. Along with *Nothochrysa*, *Dictyochrysa*, and *Hypochrysa*, whose larvae were described earlier (Box 1), *Pimachrysa* is now the fourth genus of Nothochrysinae for which larvae are known. Also, we provide new data on the larva of *Nothochrysa californica* Banks that document a broader range of larval variation than previously reported for the genus. Finally, we discuss the pattern of variation among known nothochrysine larvae in relation to

the current understanding of generic relationships and the phylogeny of the subfamily.

2. Materials and Methods

2.1. Larval Material and Procedures. Our study is based on four specimens of *Pimachrysa fusca* Adams collected in late February and late April at Lower Covington Flat, Joshua Tree National Park (previously a national monument), Riverside Co., CA. Three of the specimens (two third instars and the single second instar) were used for morphological study, and one third instar was reared to confirm the specific identity of our larval specimens. The three specimens were treated in KAAD solution [9] for 24 hours and then transferred to 95% ethyl alcohol. In the laboratory the specimens were photographed, and the external gross features were described. Subsequently, one specimen from each instar was cleared in KOH and transferred to glycerin for examination of fine structures and setation. The second instar died soon after it was collected. We noted a globule of apparently sticky material on the labrum and base of the left mandible; presumably the material was exudate from aphids that we supplied the larva while we were in the field. We were able to remove the exudate from the preserved specimen without damage.

All voucher specimens are now in the Tauber Research Collection (Lot 2015: 01); a leg from one of the third instars was given to Shaun Winterton, California Department of Food and Agriculture, Sacramento, for DNA analysis.

To rear one of the third instars to the adult stage, we held it in a glass vial provided with frozen eggs of *Ephestia kuehniella* (Zeller), the Mediterranean flour moth. It was maintained at room temperature on a long-day light: dark cycle (~LD 16:8).

2.2. Terminology and Comparisons. The chrysopid larval stage typically includes three instars, the first of which (L1, Semaphoront A) differs markedly from the latter two (L2, L3), which, in turn, usually resemble each other (Semaphoront B). The main differences between second and third instars are in size, markings, and the number of thoracic and abdominal setae. In this paper, both instars of Semaphoront B are considered.

With minor exceptions, morphological terminology and chaetotaxy followed the usage of Rousset [10], Tsukaguchi [11], Tauber et al. [12], and Monserrat and Díaz-Aranda [13]. In one case there is a discrepancy. Some abdominal segments of *Nothochrysa* have a pair of laterodorsal tubercles (LDTs); in papers by Tauber and coauthors, the term tubercle refers to a protuberance bearing more than one seta. In *Nothochrysa californica* Banks, each LDT bears one long, filiform seta and one shorter seta. Thus, the tubercles of *N. californica* are consistent with those of other chrysopids. However, third instars of the two European species of *Nothochrysa* also were reported to have laterodorsal “tubercles” on abdominal segments A5 to A7; but in these species, the “tubercles” were reported to have only one, relatively long, seta [13]. If such a situation pertains (and no small seta is found on these “tubercles”), the homology of the structures should be examined

and the usage of the term “tubercle” clarified. For convenience, in this paper, we refer to the structures reported for the European species as laterodorsal tubercles (LDTs).

For our comparisons, we used specimens of *Nothochrysa californica* Banks (all instars from Alameda and Marin Counties, California) and *Hypochrysa africana* Kimmins (second instar from South Africa, see [6]), as well as earlier descriptions in the literature, as follows: *Dictyochrysa fulva* Esben-Petersen (first instar) [14], *Hypochrysa africana* (Kimmins) (second instar) [6], *Hypochrysa elegans* (Burmeister) (first and third instars) [13, 15], *N. californica* (all instars) [16], *Nothochrysa capitata* (Fabricius) (third instar) [13, 17], and *Nothochrysa fulviceps* (Stephens) (first and third instars) [13, 17]. Our comparisons are summarized in Box 2.

3. Background

3.1. Habitat. The collection site (northwestern corner of Joshua Tree National Park) is in a high elevation portion of the Mohave Desert (elevation: ~4670 ft). The area is open and arid, with scattered, low-growing trees and bushes amongst rocky outcroppings. Vegetation at the site includes *Quercus cornelius-mulleri* (Muller oak), *Juniperus californica* (California juniper), *Pinus monophylla* (single-leaf pinyon pine), *Larrea tridentata* (creosote bush), *Bebbia juncea* (Bebbia), *Yucca brevifolia* (Joshua tree), and *Yucca schidigera* (Mohave yucca). Our specimens were beaten from oak bushes, *Q. cornelius-mulleri*. The collections occurred on two days: the first (a single second instar, L2) on 26-2-2015 (cool and sunny), and the second (three third instars, L3) on 28-4-2015 (warm and sunny).

3.2. Larval Behavior. The specimens did not carry debris and they gave the overall impression of a *Chrysoperla* larva, but their bodies appeared thicker and more rounded (Figures 1(a)–1(c)). They remained motionless during much of the time we observed them; occasionally they moved quickly. The third instars, in the laboratory, fed readily on eggs of *E. kuehniella*; one specimen spun a delicate, oval shaped cocoon on the side of its vial 5-6 days after captivity; small pieces of debris were incorporated into the outer layers of the cocoon. The larva (within the cocoon) remained in diapause for 4 to 5 months, pupated ~155 days after spinning, and emerged as an adult 15 days later. The pharate adult exited the cocoon via a circular opening that it cut at the head end of the cocoon.

3.3. Generic Diagnosis. Our *P. fusca* larvae have the three diagnostic features that typify nothochrysine larvae. (i) The flagellum is very short relative to the pedicel (~1/14th the length of the pedicel) (Figure 1(d)). (ii) The terminus of the flagellum has a group of short apical setae. (iii) More than three lateral sensilla are present on the terminal segment of the labial palps (L2 = 5, L3 = 4-5) (Figure 1(e)).

Among the Nothochrysinæ with known larvae, *Pimachrysa* (*P. fusca*) closely resembles *Hypochrysa* (two species) (Box 2). That is, the larvae (second and third instars) from both genera have elongate, naked bodies; the thorax and abdomen lack lateral tubercles; the abdomen has no laterodorsal tubercles. In addition, none of the setae are

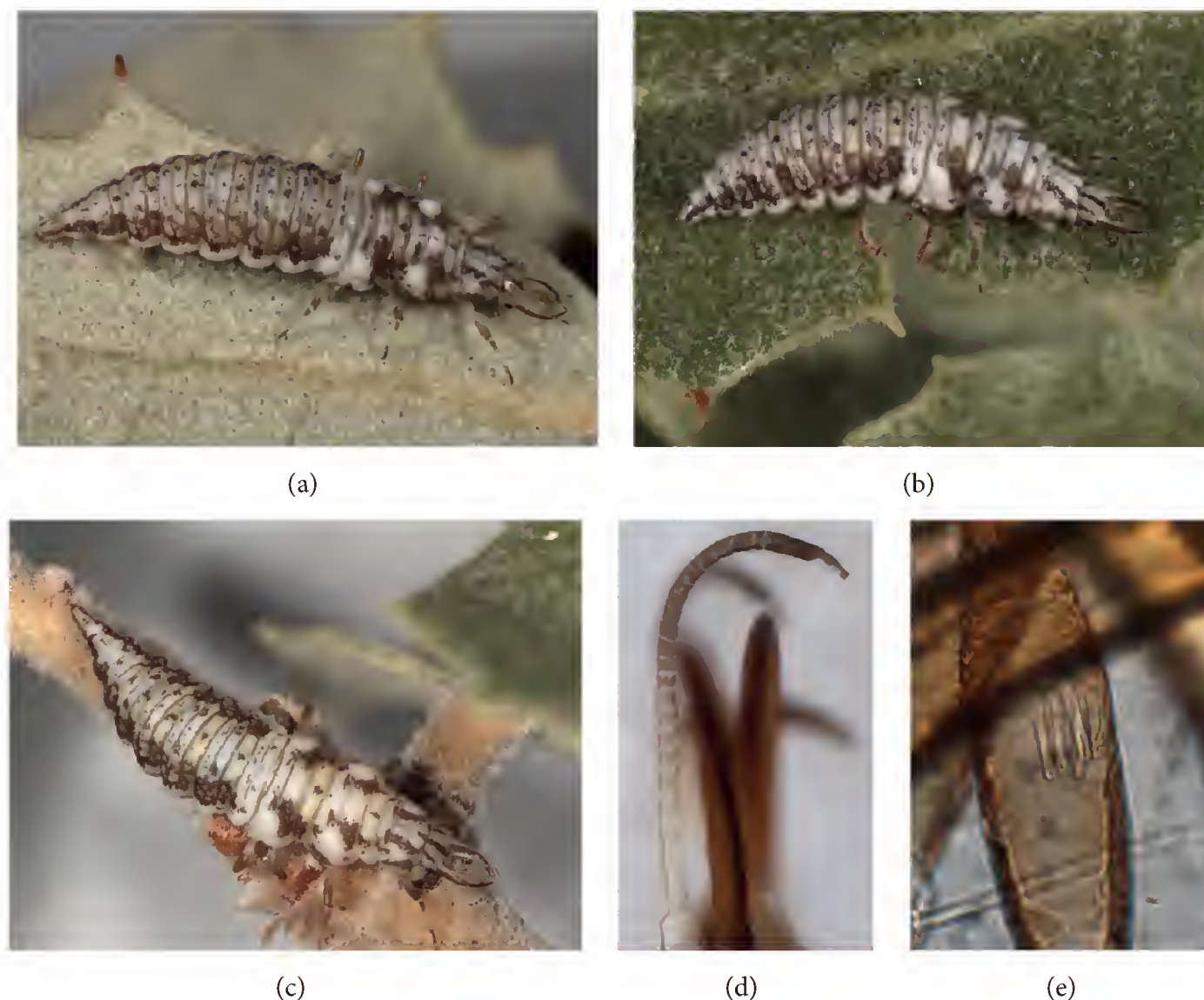


FIGURE 1: *Pimachrysa fusca* Adams, Joshua Tree National Park, Riverside Co., CA. (a, b, and c) Body, dorsal, illustrating the variation in the dorsal body markings among three third-instar specimens collected at the same locality on the same day. (d) Antenna (second instar), lateral, showing foreshortened flagellum typical of nothochrysine larvae. (e) Labial palps (second instar), lateral, showing row of five sensilla on terminal segment.

hooked, and the primary cephalic setae of the *Pimachrysa* larvae are slightly blunt and thus similar to those of *Hypochrysa*. Finally, the *Pimachrysa* head and body markings resemble those of *H. elegans*; that is, larvae of both species have dark brown, elongate, dorsal head markings, and dark brown, sublateral stripes on the thorax, and abdomen (most noticeable on the L3; see Figures 1(a), 1(c), 2(a), 2(b), and 6).

However, a number of distinguishing features differentiate the larvae of the two genera. First, other than the cephalic setae, all other setae on the *Pimachrysa* larvae, especially the abdominal submedian setae (SMS), are short and acutely tipped, whereas on *Hypochrysa* they are short and blunt or clavate. Second, the jaws of *Hypochrysa* are longer than those of the *Pimachrysa* (*Hypochrysa*: 1.7–1.8x head length; *Pimachrysa* 1.4–1.6x head length). Third, the white-to-tan body coloration of the *Pimachrysa* larvae differs markedly from the green-to-bright green coloration of both *H. elegans* and *H. africana*. In our opinion, the above differences offer significant supporting evidence for the separate identity of the two genera.

4. Description of *Pimachrysa fusca* Adams

4.1. Second Instar

4.1.1. *Body* (Figures 2(a)–2(c)). Length ~5.5 mm (measured in lateral view through spiracles), height ~0.94 mm (thickest

section of abdomen). Cream-colored-to-white with dark brown dorsolateral and lateral markings on each segment; lateral surface with brown dorsal band above lateral band of white and lower narrow band of dark brown; pronotal sclerites brown. All setae short, smooth, pale, of two types: “simple” = with acute tip (most setae) or “slightly blunt” = with slightly rounded tip (some primary cranial setae). Thoracic, abdominal setae often with swollen, lightly sclerotized setal bases (Figures 3(a) and 3(c)), these setal bases becoming increasingly larger and more sclerotized posteriorly.

4.1.2. *Head* (Figures 2(a)–2(c), 4(a), and 4(b)). Dorsum white, with dark brown markings as in figures; frontal marking elongate, without lateral arms; mesal epicranial and/or postfrontal markings reduced to pair of posterior spots below frontal marking; lateral epicranial marking elongate, extending to lateral side of antennal base; eyes with stemmata clear, integument between stemmata, surrounding all stemmata dark brown to black; cranium roughly triangular, with posterior margin (dorsal) rounded, ventral margin elongate; width (across eyes) ~0.79 mm, length (dorsum) ~0.64 mm, depth (midregion to top of eye) ~0.20 mm; base mostly exposed. Clypeolabral area elongated distally, extending over basal segment of labial palps; with large brown mark mesally. All dorsal primary cephalic setae present except S10 and S12; S11 long, S3 short and shifted mesally, others of medium length, most slightly blunt apically; Vx setae obscured by cervix, probably

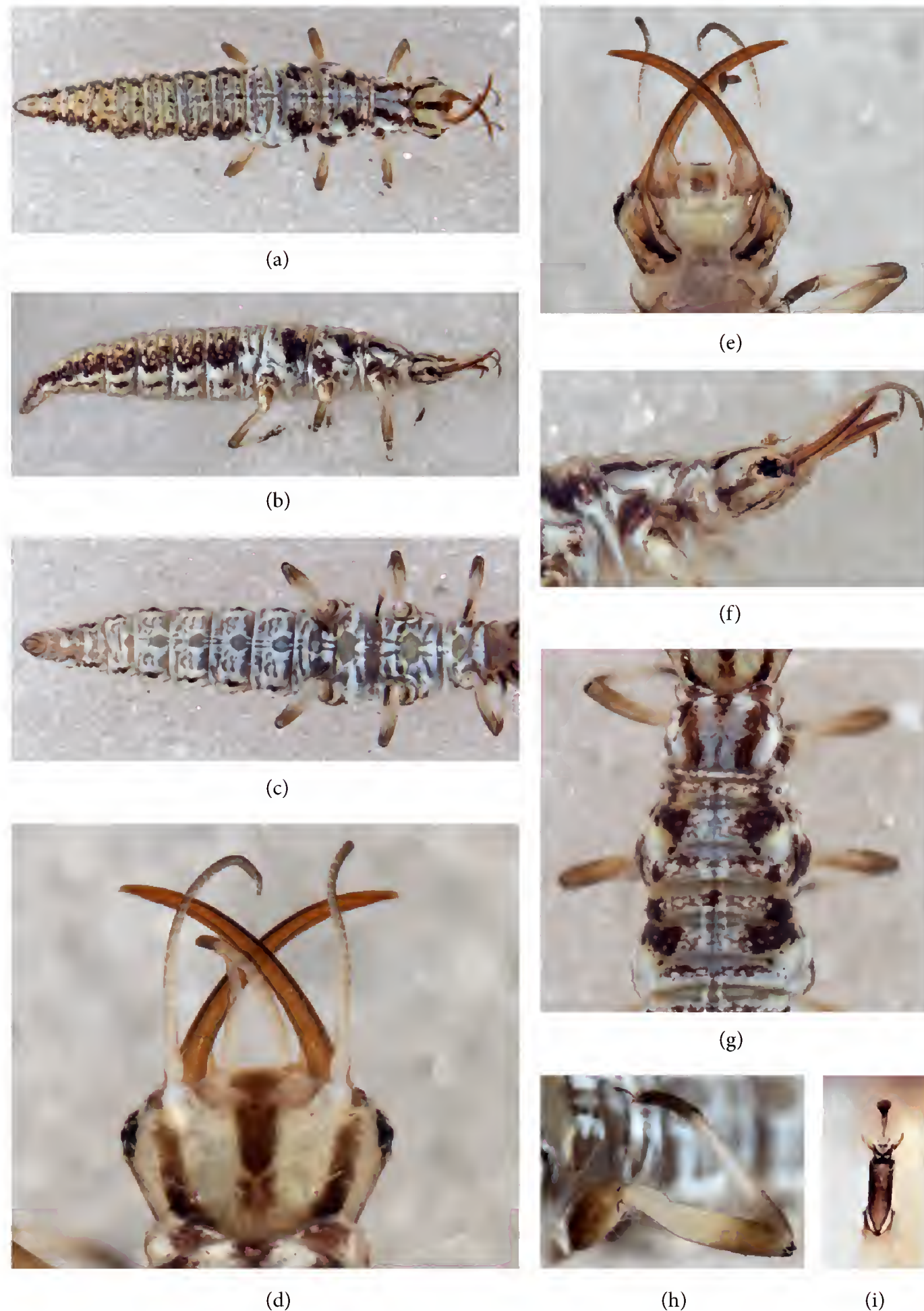


FIGURE 2: *Pimachrysa fusca* Adams, second instar (Joshua Tree National Park, Riverside Co., CA). (a) Body, dorsal. (b) Body, lateral. (c) Body, ventral. (d) Head, dorsal. (e) Head, ventral. (f) Head, lateral. (g) Thorax, dorsal. (h) Hind leg, anterior. (i) Tarsus, dorsal.

with three small setae (as drawn on Figure 4(a)) or two setae and pore; labrum with three pairs of setae (one mesally, two laterally); dorsum without secondary setae.

Cervix full laterally, ventrally, slightly withdrawn from cranium dorsally; with row of 6–8 pairs of setae along anterior margin, dorsum to venter.

4.1.3. Cephalic Appendages (Figures 2(d)–2(f), 4(a), and 4(b)). Mandible long, thin, length ~ 0.88 – 0.90 mm (along curved exterior margin), length 0.74 – 0.78 mm (straight line), width

(at base) 0.10 – 0.13 mm; ratio mandible length (curved margin) to head width = 1.11 – 1.13 ; ratio mandible length (curved margin) to head length (dorsal) = 1.37 – 1.40 . Mandible very slightly upturned distally, with single acute basolateral seta; terminus sharp, with four very small teeth. Antennal length 0.94 mm, ~ 1.5 x length of cranium; width ~ 0.04 mm (at widest part of pedicel); scape with relatively straight sides, two pairs of distal setae (one lateral, other dorsal); pedicel annulate, with ~ 18 – 22 medium-length annulations mesally, one elongate annulation basally, one distally, distal annulations with two small setae, one lateral, one mesal, distomesal margin

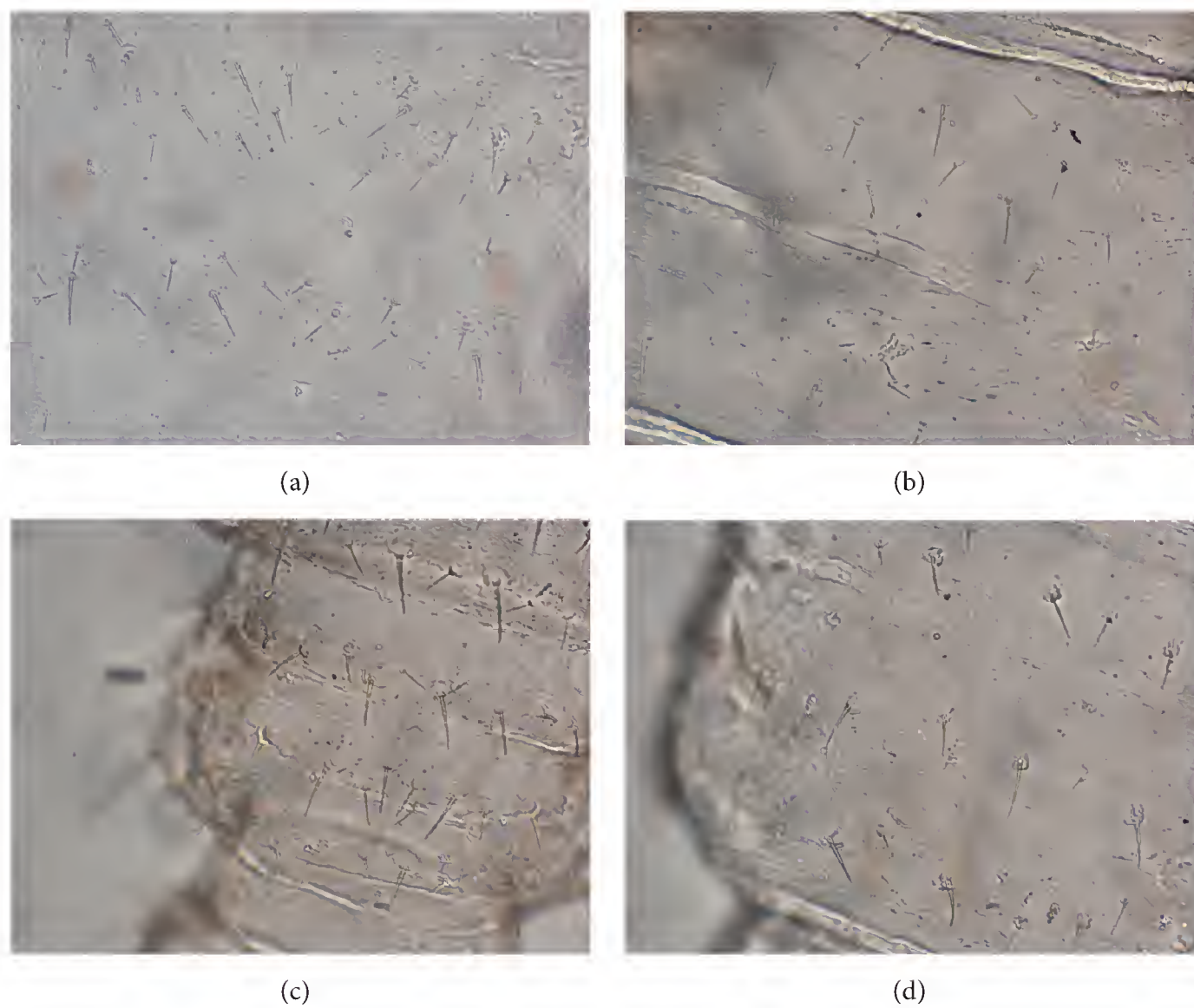


FIGURE 3: *Pimachrysa fusca* Adams (Joshua Tree National Park, Riverside Co., CA), thoracic (a, b) and abdominal (c, d) integument and setae, dorsal. Note slight differences in size of setae and chalazae between second (a, c) and third (b, d) instars. All images are the same magnification ($\times 100$).

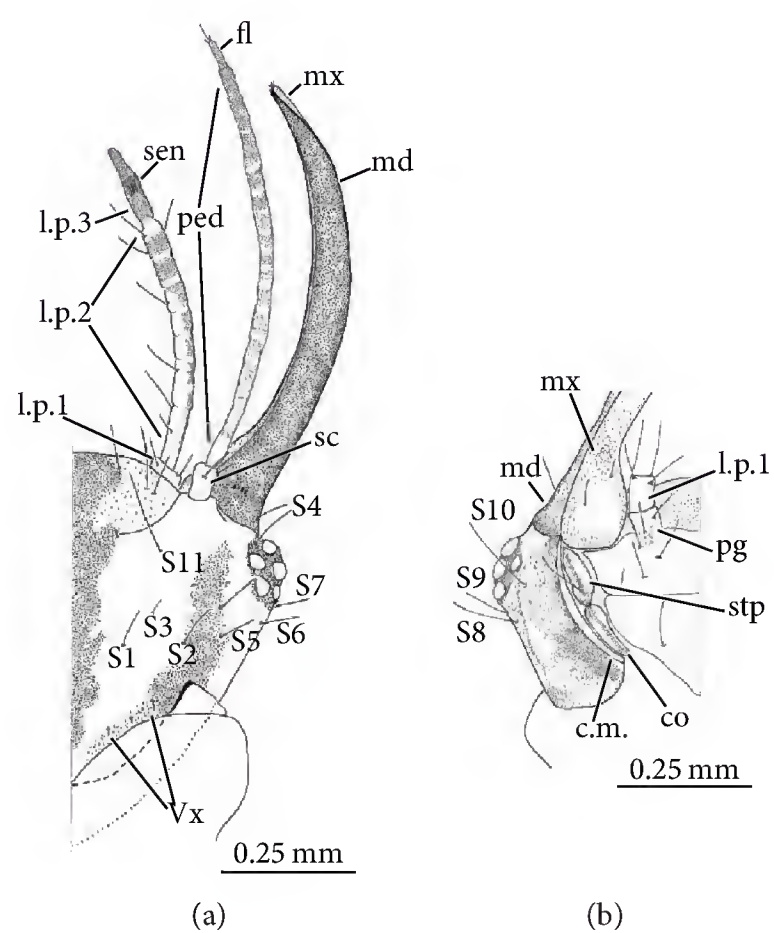


FIGURE 4: Head of *Pimachrysa fusca* Adams, second instar. (a) Dorsal. (b) Ventral. co: cardo; c.m.: cranial margin; fl: flagellum; l.p.x: labial palps, number of segment; md: mandible; mx: maxilla; ped: pedicel; pg: palpiger; sc: scape; stp: stipes; Sx: primary seta number; Vx: Vx setae.

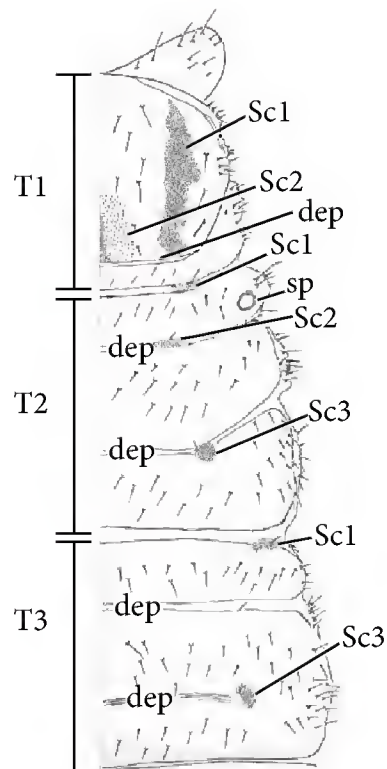


FIGURE 5: Thorax (dorsal) of *Pimachrysa fusca* Adams, second instar. dep: smooth-surfaced, intrasegmental depression between subsegments; Sc1, Sc2, and Sc3: first, second, and third primary sclerites of each segment; sp: spiracle; Tx: number of thoracic segment.

concave, bearing small claw; flagellum short, stubby, ~ 0.08 times length of pedicel, terminus with one short apical seta, surrounded by three or four microsetae. Labial palp long, slender, ~ 0.85 times length of mandible (straight line); basal segment with four distal setae, two dorsally, two ventrally; middle segment long, with long, undivided basal subsegment bearing $\sim$ three setae mesally, with $\sim$ six mesal subsegments, one mesal seta; terminal subsegment elongate, with two long setae distally, one basally; terminal labial segment $\sim$ one-third length of middle segment, slightly tapered distally, heavily sclerotized, no setae observed, ventrolateral margin with five closely spaced, elongate sensilla. Palpiger erect, with relatively straight sides, one mesal seta, one lateral seta; prementum broad, with three pairs of setae; cardo, stipes elongate, narrow, longitudinally arranged, cardo behind stipes, stipes with long seta.

4.1.4. Thorax (Figures 2(g) and 5). Lateral tubercles absent; dorsum with scattered, short, acute setae; pleurum with longitudinal, dorsal band of short bristles, spines; primary setae unidentified (except as noted below and in Figure 5). Legs (Figures 2(h) and 2(i)) white, with white-to-cream-colored setae; coxa dark brown; distal one-third of femur, basal one-fifth of tibia dark brown, with margins of all adjoining segments black distally; tarsus, claw, empodium dark brown.

Prothorax (T1) with two well-delineated subsegments separated by transverse depression. Paired Sc1 dark brown, elongate, extending full length of anterior subsegment, slightly narrow anteriorly, broader mesally, posteriorly, underlaid by dark brown, pigmented lateral stripe. Sc2 broadly ovate, extending from middle to posterior margin of anterior subsegment, with longitudinally elongate, brown marking posteromesally.

Mesothorax (T2) consisting of three well-delineated subsegments separated by distinct depressions. Anterior subsegment with pair of ovate sclerites (Sc1) on anterior margin, with single lateral seta (probably S1Sc1); spiracles simple, sessile, circular, brown, with cylindrical atrium. Second subsegment with pair of small sclerites (Sc2) on anterior margin, each with single seta (S1Sc2), juxtaposed much smaller seta (S2Sc2); subsegment separated mesally from posterior subsegment by transverse depression with pair of large, brown, circular sclerites (Sc3) laterally, each with small mesal seta (S1Sc3).

Metathorax (T3) consisting of three well-delineated subsegments separated by transverse depressions, each with pair of small setae laterally (probably S1Sc1, S1Sc2), but sclerites Sc1, Sc2 not identified. Sc3 medium-sized, brown, circular, with small mesal seta (S1Sc3).

4.1.5. Abdomen (Figures 2(a), 2(b), 2(c), and 6). Lateral tubercles (LTs), laterodorsal tubercles (LDTs) absent; spiracles (A1–A8) circular, sessile, with simple atrium. Each segment (A1–A7) roughly quadrangular, divided into three subsegments (or raised folds) separated by transverse depressions, anterior one extending to margin of segment; spiracle located laterally on dorsum of second subsegment; dorsum of all subsegments with acute submedian setae (SMS) on chalazae of small to medium sizes, arranged in transverse rows on each subsegment; pleura with patches of small, mostly acute, sometimes blunt setae, spines. A1: anterior subsegment short, dorsum with four pairs of SMS; second, third subsegments longer, similar in size to each other, each with row of $\sim$ ten to twelve pairs of SMS, most stemming from relatively small chalazae. A2–A7: dorsum of each subsegment with $\sim$ nine to twelve pairs of SMS; number and length of SMS, size (plumpness) of chalazae becoming progressively larger posteriorly. A7: first subsegment with four pairs of short SMS stemming from relatively large, plump chalazae; second and third subsegments each with five to seven pairs of SMS, most with plump chalazae. A8: segment divided into three subsegments; anterior subsegment narrow, well-delineated from subsequent subsegment; second, third subsegments not well-delineated from each other; dorsum of anterior subsegment with one pair of SMS, middle subsegment with two pairs of SMS; posterior subsegment with $\sim$ seven pairs of SMS; pleural region of segment with two, unsclerotized, lateral swellings, each bearing $\sim$ five medium length setae. A9: cylindrical, with two visible subsegments; anterior subsegment without setae anteriorly, with $\sim$ three pairs of robust dorsal setae posteriorly, lateral-most pair on swelling; posterior subsegment with row of $\sim$ seven pairs of medium length setae on robust chalazae posteriorly. A10: dorsum with $\sim$ six pairs of setae laterally, $\sim$ eight pairs of setae extending from posterior membrane on terminus. Venter with subsegmentation poorly demarcated; each segment (A1–A8) with two transverse bands of acute setae and spines (microsetae), pair of large, sublateral patches of relatively robust setae and spines (microsetae); setae often longer than those on dorsum, but without enlarged chalazae.

4.2. Third Instar. Similar to second instar except for measurements and noteworthy features, as follows.

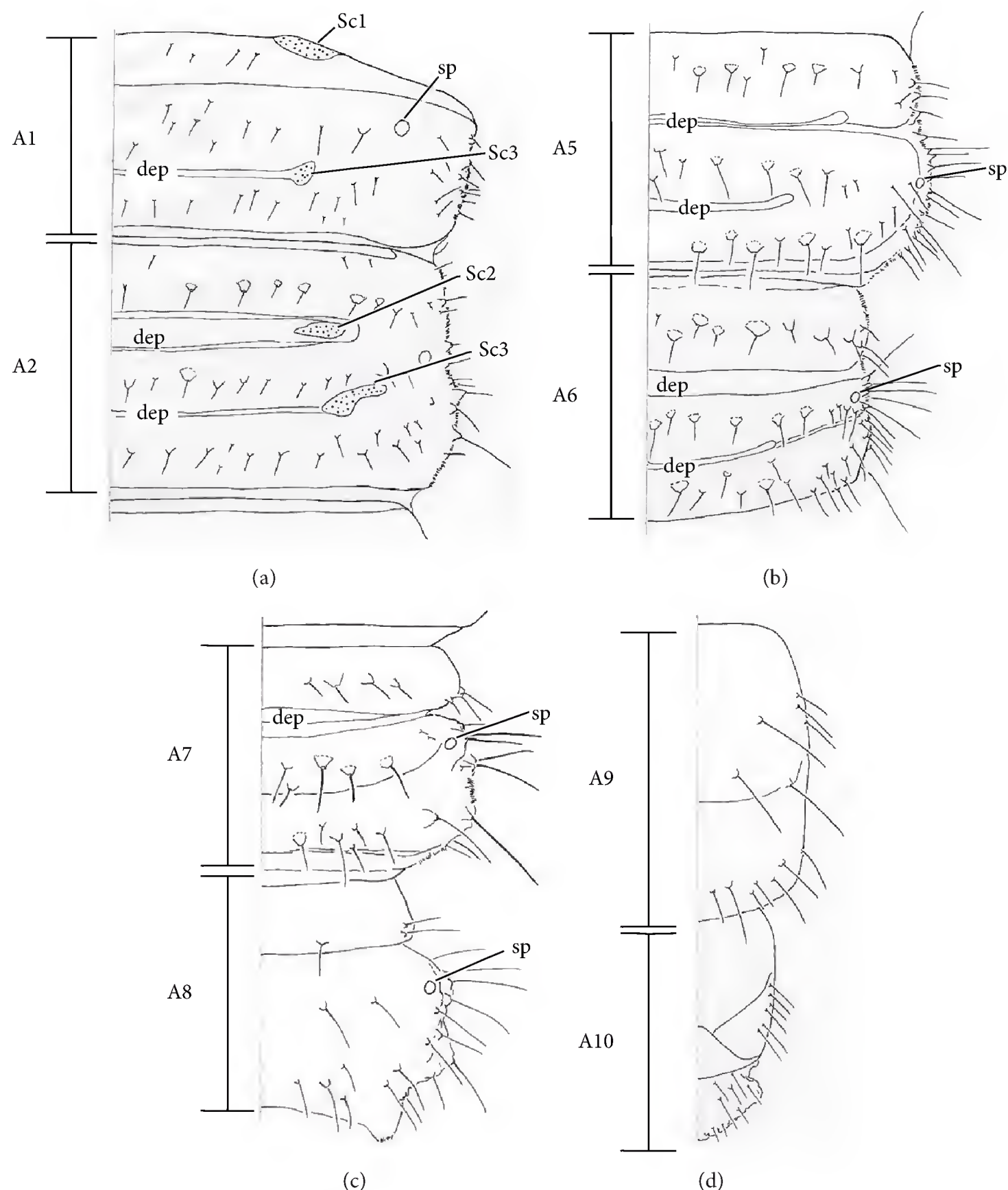


FIGURE 6: Abdomen (dorsal) of *Pimachrysa fusca* Adams, second instar. (a) First and second segments. (b) Fifth and sixth segments. (c) Seventh and eighth segments. (d) Ninth and tenth segments. Ax: number of abdominal segment; dep: smooth-surfaced, intrasegmental depression between subsegments; Sc1, Sc2, and Sc3: first, second, and third primary sclerites of each segment; sp: spiracle.

4.2.1. Body (Figures 1(a)–1(c), 7, 8(a), and 8(b))

Body length (measured in lateral view through spiracles) 8.5–9.0 mm (L2: 5.5 mm).

Body depth (thickest) 1.5–1.6 mm (L2: ~0.94 mm).

Subsegmentation, setation, coloration as in L2.

Setal size approximately same as L2. Thoracic, abdominal setal bases sometimes slightly swollen, sclerotized (Figures 3(b) and 3(d)).

4.2.2. Head (Figures 7(a) and 8(c))

Head width (across eyes) 0.77–0.78 mm (L2: 0.79 mm).

Head length (dorsum) 0.63–0.64 mm (L2: ~0.64 mm).

Head depth (midregion to top of eye) 0.18–0.19 mm (L2: ~0.20 mm).

4.2.3. Cephalic Appendages (Figures 7(a) and 8(c))

Mandible length (along curved exterior margin) 0.93–0.97 mm (L2: ~0.88–0.90 mm).

Ratio, mandible length to head width = 1.19–1.29 : 1 (L2: 1.11–1.13 : 1).

Ratio mandible length to head length (dorsal) = 1.46–1.57 : 1 (L2: 1.37–1.40).

Antennal length ~0.96 mm, ~1.5x length of cranium (L2: ~0.94 mm, ~1.5x length of cranium).

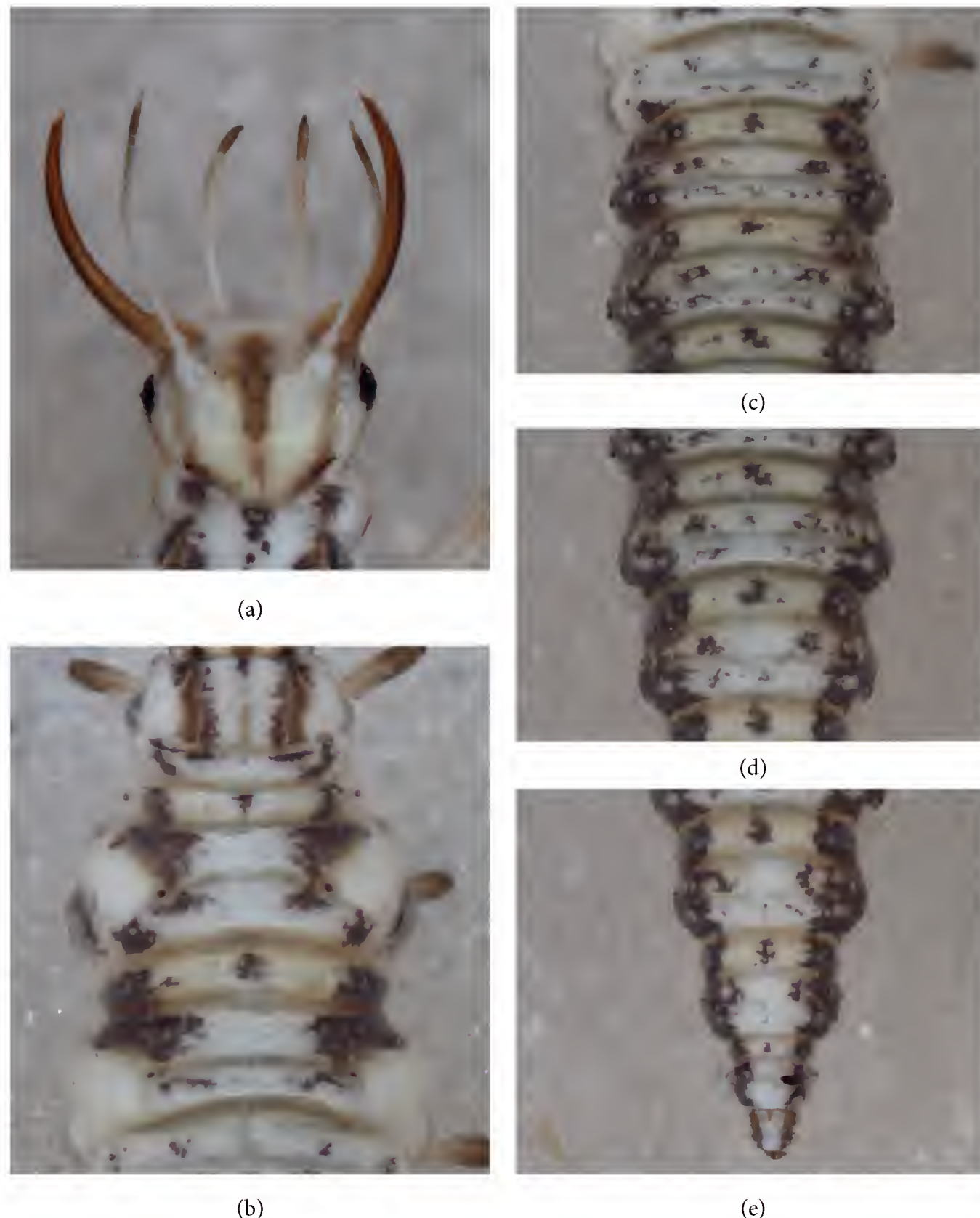


FIGURE 7: *Pimachrysa fusca* Adams, third instar (Joshua Tree National Park, Riverside Co., CA). (a) Head, dorsal. (b) Thorax, dorsal. (c) Abdominal segments 1 through 3, dorsal. (d) Abdominal segments 4 and 5, dorsal. (e) Abdominal segments 6 through 10, dorsal.

Flagellar length 0.06-0.07 mm (L2: 0.06 mm); terminus with single very short apical seta, few, if any, surrounding microsetae.

Ratio, length of flagellum : length of pedicel 0.08 : 1 (L2: 0.07 : 1).

Ratio, length of labial palp : length of mandible (straight line) ~0.78 : 1 (L2: ~0.85 : 1).

Labial palp: middle segment with ~seven subsegments; ventrolateral margin of terminal segment with four to five closely spaced, elongate sensilla.

4.2.4. Thorax (Figures 7(b) and 8)

Legs as in L2, except coxa white distally; basal one-third of tibia dark brown.

4.2.5. Abdomen (Figures 7(c)–7(e), 8(a), and 8(b)). As in L2, with following exceptions:

A1: anterior subsegment dorsally with eight pairs of SMS; second, third subsegments each with row of ~twelve to fifteen pairs of SMS, most stemming from relatively small chalazae.

A2–A7: dorsum of each subsegment with ~nine to fifteen pairs of SMS—one to three more than L2.

A8: dorsum of anterior subsegment with two pairs of SMS; pleural region with single pair of broad, unsclerotized, lateral swellings, each bearing ~ten setae.

A10: basal portion of segment withdrawn and shorter than on L2.

Venter similar to L2.

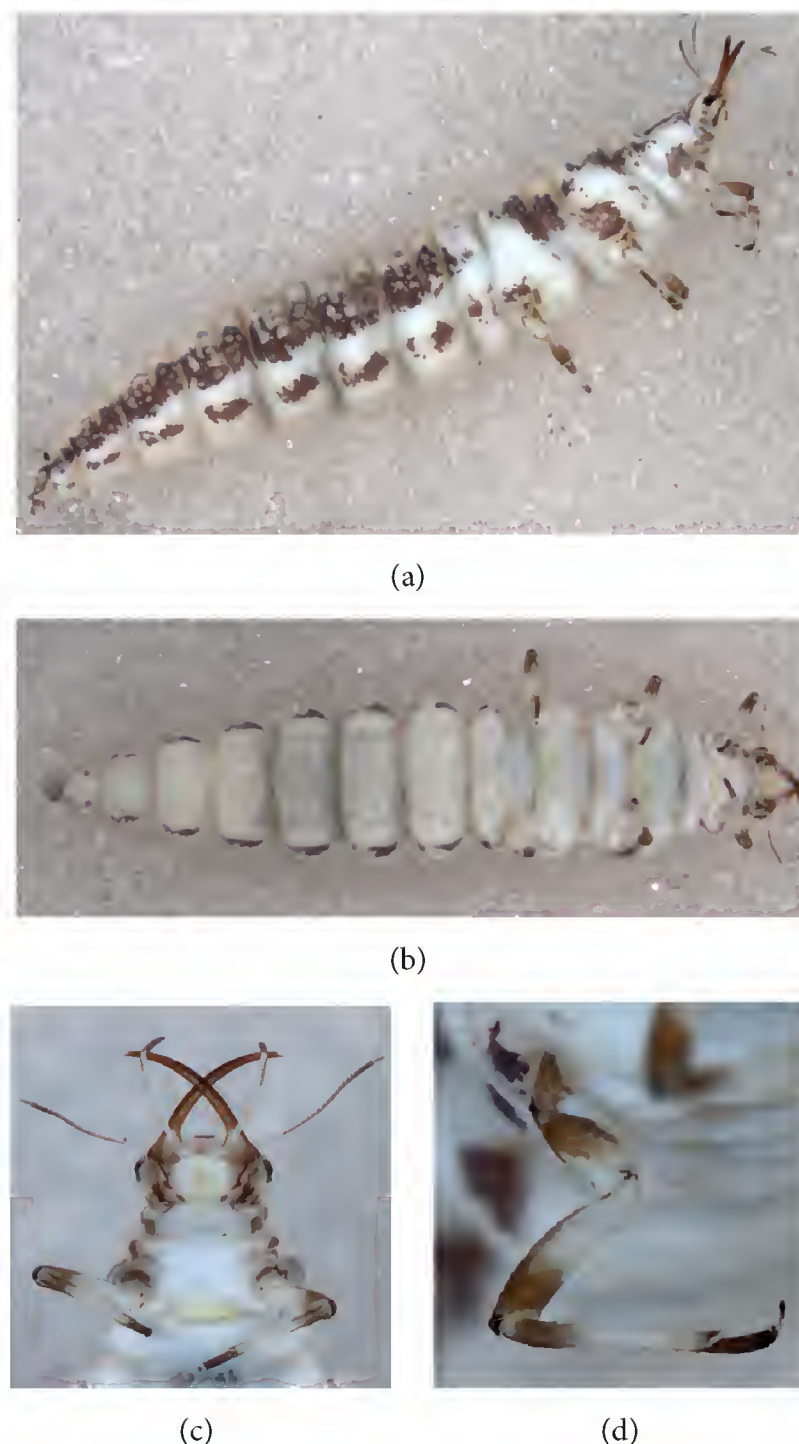


FIGURE 8: *Pimachrysa fusca* Adams, third instar (Joshua Tree National Park, Riverside Co., CA). (a) Body, lateral. (b) Body, ventral. (c) Head, prothorax, ventral. (d) Hind leg, anterior.

4.3. Size Differences between Second and Third Instars. Among our specimens, the L2 ($n = 1$) and L3 ($n = 2$) express some unusual variation in the relative sizes of body parts. The L3s, as expected, have much larger bodies than the L2, and yet the head size is almost identical among our specimens of the two instars. Also, while the L3 mandibles appear somewhat larger than those of the L2, the antennae and labial palps do not. In addition, the L3 setal size is approximately the same as in the L2, but the degree of enlargement and sclerotization of the abdominal setal bases is less than on L2 (Figures 3(a) and 3(c) versus 3(b) and 3(d)). Thus, the L3 setae seem less prominent than those of the L2 (Figure 3).

Given the small number of specimens, we are puzzled by the above variation in the relative sizes of larval body parts. First, we do not know whether our observations represent the normal situation for this species. Second, if it does, we suggest that the variation could be related to some unusual pattern of annual or seasonal variation in the availability of food or perhaps seasonal or generational differences in physical factors.

4.4. Cocoon (Figures 16(a) and 16(b)). White, oblong longitudinally, round transversely, with basal side spun directly against substrate; attached base with single layer of thin, transparent silk; upper surface with two layers of silk: inner layer: dense, relatively smooth and outer layer: sparse, mixed with debris.

5. Comparative Information on *Nothochrysa californica* Banks

Previously, the three instars and cocoon of *N. californica* were described in detail [16]. However, full comparisons with other described nothochrysinine immatures are hampered because the descriptions lack adequate illustrations and several larval characters were not included. Here, we provide images and drawings of the *N. californica* second instar (Figures 9–13) and the following notes on all instars for use in comparisons. All observations and measurements below are based on one to two specimens per instar:

Mandible length (along curved exterior margin) = L3: 1.63–1.66 mm; L2: 1.15 mm; L1: 0.80 mm.

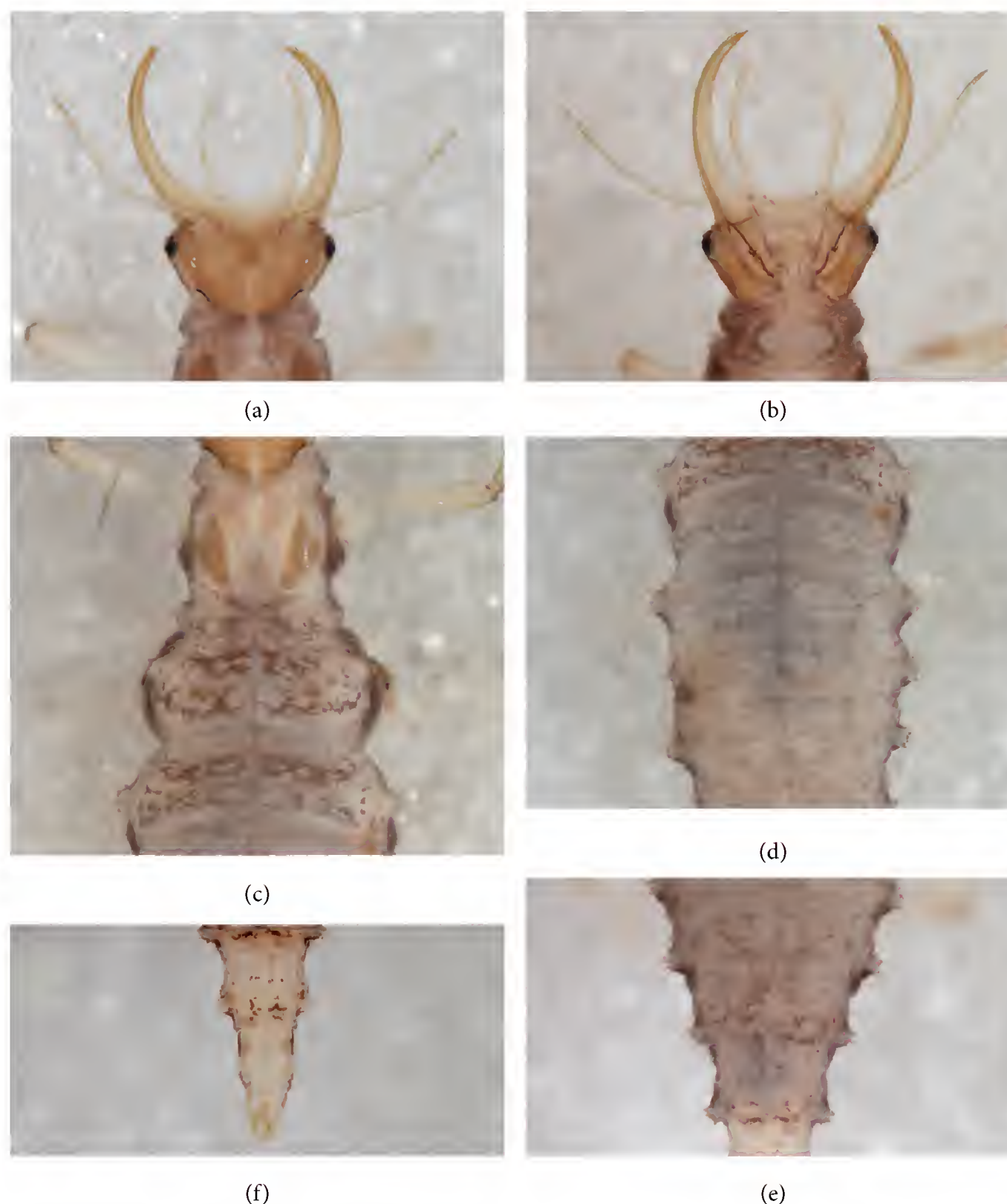


FIGURE 9: *Nothochrysa californica* Banks, second instar (Strawberry Canyon, University of California Campus, Alameda Co., CA). (a) Head, dorsal. (b) Head, ventral. (c) Thorax, dorsal. (d) Abdominal segments 1 through 4, dorsal. (e) Abdominal segments 5 through 7, dorsal. (f) Abdominal segments 8 through 10, dorsal.

Antennal length = L3: ~1.65 mm, 1.5x length of cranium; L2: 1.24 mm, 1.8x length of cranium; L1: 0.84 mm, 1.6x length of cranium.

Pedicel with claw at tip = L3: yes; L2: yes; L1: yes.

Pedicel length = L3: 2.16 mm; L2: 0.97 mm; L1: 0.58 mm.

Flagellar length = L3: 0.18 mm; L2: 0.17 mm; L1: 0.17 mm.

Length of flagellar seta = L3: 0.03 mm; L2: 0.03 mm; L1: 0.04 mm.

Labial palp, number of lateral sensilla on terminal segment = L3: 4; L2: 4-5; L1: 0.

6. Unique Larval Characters for Nothochrysinæ

Three larval features have been proposed as unique, and perhaps apomorphic, for the subfamily Nothochrysinæ [5, 13, 18]. Given the recent data from *Hypochrysa* and now *Pimachrysa*, as well as our reexamination of the *N. californica* larvae, we were led to reevaluate the diagnostic and phylogenetic value of these characters. In addition, we propose an addition larval character that may be unique to the Nothochrysinæ. For our larval comparisons, we used our own measured values and estimated others from published drawings (*Dictyochrysa*: Figure 3 by New [14]; *N. capitata*, *N. fulviceps*: Figures 46 and 47 by Monserrat and Díaz-Aranda [13]).



FIGURE 10: *Nothochrysa californica* Banks, second instar (Strawberry Canyon, University of California Campus, Alameda Co., CA). (a) Body, dorsal. (b) Body, lateral. (c) Body, ventral.

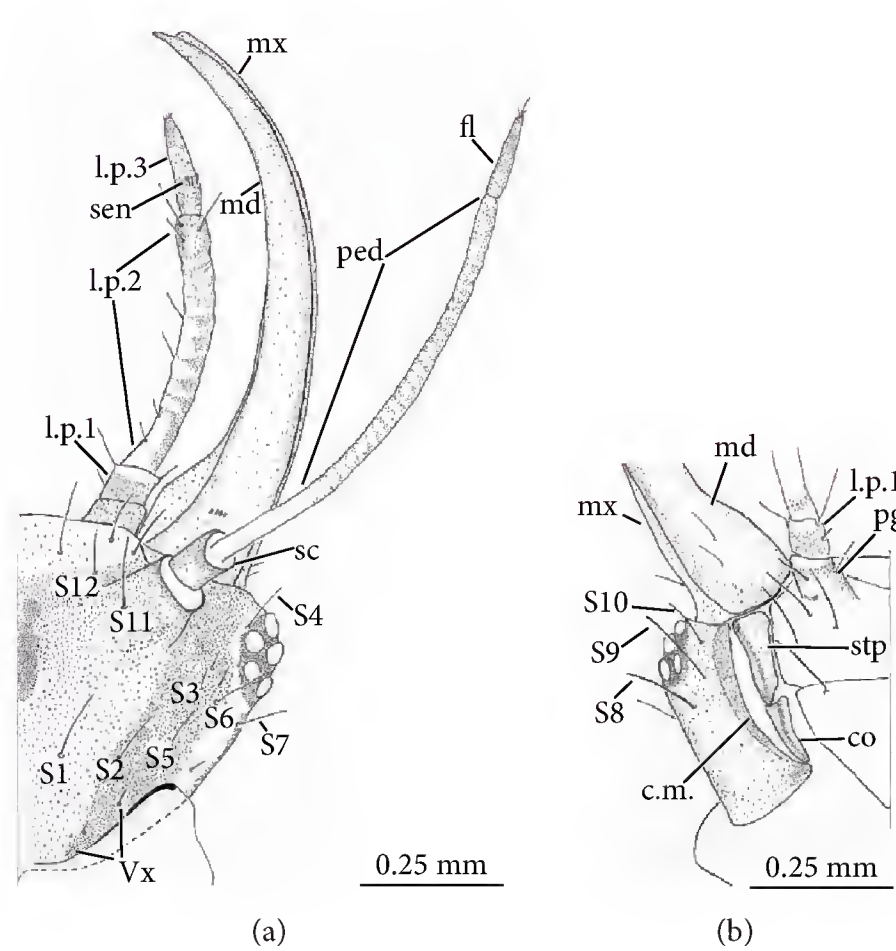


FIGURE 11: Head of *Nothochrysa californica* Banks, second instar. (a) Dorsal. (b) Ventral. co: cardo; c.m.: cranial margin; fl: flagellum; l.p.1–3: labial palps, number of segment; md: mandible; mx: maxilla; ped: pedicel; pg: palpiger; sc: scape; sen: sensilla; stp: stipes; Sx: primary seta number; Vx: Vx setae.

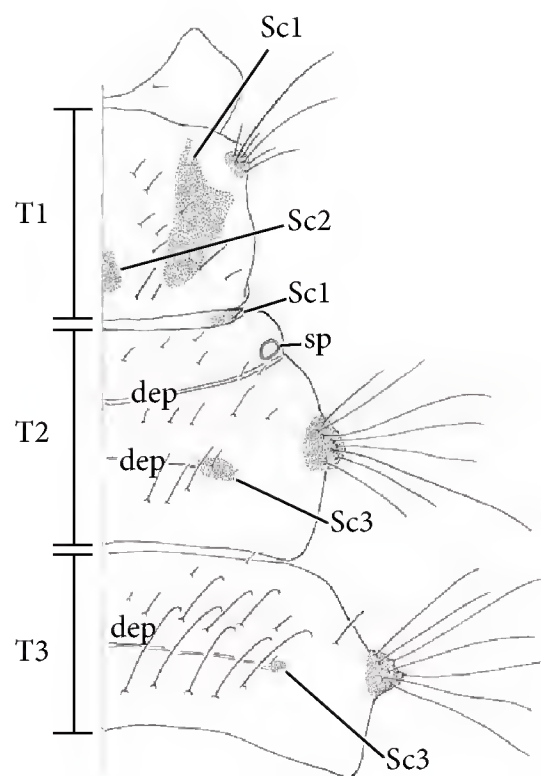


FIGURE 12: Thorax (dorsal) of *Nothochrysa californica* Banks, second instar. dep: smooth-surfaced, intrasegmental depression between subsegments; Sc1, Sc2, and Sc3: first, second, and third primary sclerites of each segment; sp: spiracle; Tx: number of thoracic segment.

6.1. Length of Flagellum. The nothochrysine antenna is characterized as having a terminal segment (flagellum) that typically is short and stubby, that is, approximately 10 or more times shorter than the pedicel; in larvae of Apochrysiinae and Chrysopinae, the flagellum is considerably longer—only about 3–6 times shorter than the pedicel. For *Hypochrysa* and *Pimachrysa* second and third instars, as well as *N. fulviceps* and *N. californica* third instars, this character is consistent (their flagella are ~12–20 times shorter than the pedicel). Also, for the *Dictyochrysa* first instar, it is close at ~9 times shorter than the pedicel. However, there are interesting examples of interspecific and developmental variation in *Nothochrysa*. For example, the third instar of *N. fulviceps*, with a flagellum that is 12.5 times shorter than the pedicel, is consistent with the character, and the third instars of both *N. capitata* and *N. californica* (with flagella 8.3–9.1 times shorter than the pedicel) are near the 10x designation (Box 2). However, the flagella of the first and second instars of *N. californica* are noticeably longer, with the first instar being the most deviant (L3: flagellum 9.4–9.5 times shorter, L2: 5–7 times shorter, L1: only 4 times shorter than the pedicel; see Figure 14). Whether this pattern of developmental variation applies to the first instars of the European *Nothochrysa* species is unknown. However, if the old maxim “ontogeny recapitulates phylogeny” holds for this genus, the pattern could have phylogenetic importance.

6.2. Terminal Flagellar Setae. The nothochrysine flagellum is typified as bearing several, very small terminal setae; in contrast, the tip of the flagellum in Chrysopinae and Apochrysiinae bears a single long seta with a robust base. Upon reevaluation, this trait holds for most of the known nothochrysine larvae—*Dictyochrysa* (L1, one species), *Hypochrysa* (L1, L2, and L3, one species each), *Pimachrysa* (L2, L3, one species,

Figure 14(a)), and *Nothochrysa* (L3, two European species). However, the structure differs in *N. californica*; in this species the flagellum terminates with a single elongate seta having a broad base (Figure 14(b)). The length of the terminal seta varies from 0.18 to 0.25 times the length of the flagellum. Again, the deviation from the typical nothochrysine pattern is most pronounced in the *N. californica* first instar. Unfortunately, the situation with the first and second instars of the European species is unknown.

6.3. Number of Sensilla on Terminal Segment of Labial Palp. Thus far, this character appears to be a consistent means of distinguishing nothochrysine larvae (second and third instars, Semaphoront B; there are no sensilla on the palps of the first instar). A number of four or more sensilla is typical of nothochrysine larvae. Three or fewer sensilla are present on larvae of Apochrysiinae and Chrysopinae (note: this character is difficult to examine; it requires careful placement and often manipulation of a cleared specimen with labial palps in ventrolateral position and under sufficient lighting.)

6.4. Cocoon Structure. As described here, the *Pimachrysa* cocoon is sessile and strongly oblong; the basal membrane of the cocoon is single, thin, and highly transparent, whereas the upper layer is double, thick, and opaque. Among the Nothochrysiinae, this cocoon structure is shared with *Nothochrysa* (Toschi 1965: *N. californica*); however, the situation is not clear for *Hypochrysa*. *Hypochrysa africana* shares the oblong (“barrel”) shape of *Pimachrysa* and *Nothochrysa* (Duelli et al. 2010, as *Kimochrysa*), but it is unknown if its cocoon is sessile or entire. In any case, the *Pimachrysa* and *Nothochrysa* cocoons appear unlike the entire, more spherical cocoons spun by larvae of many Chrysopinae (Chrysopini, Leucochrysiini) that we have observed.

6.5. Synapomorphies? Whether the above characters constitute apomorphies or symplesiomorphies is unconfirmed, and available evidence from the hemerobiids (the presumed sister group of Chrysopidae) is inconclusive. For example, among the hemerobiids, (1) the length of the flagellum exhibits intergeneric variability: in *Boriomyia fidelis* (Banks), the flagellum is short relative to the pedicel [19]; in *Hemerobius* species, it is elongate [20]; in *Micromus* species, it is indistinguishable from the pedicel [20]. (2) The length of the terminal flagellar seta also shows variability among hemerobiid genera: in *B. fidelis* and *Drepanacra binocula* (Newman) the flagellum is elongate [19, 21]; in *Micromus* it is short and accompanied by a very small seta [20]; in *Hemerobius* it is also short, but without a small seta [20]. To our knowledge, no hemerobiids have been shown to have a cluster of very short setae on the terminal antennal segment as found in *Hypochrysa*, *Pimachrysa*, and *Dictyochrysa*. (3) To our knowledge, lateral sensilla have not been reported from the terminal segment of hemerobiid labial palpi. They appear to be unique to Chrysopidae; however, the plesiomorphic number is unknown. (4) At this point, reports on the structural features of Neuropteran cocoons are not sufficient for meaningful phylogenetic comparisons.

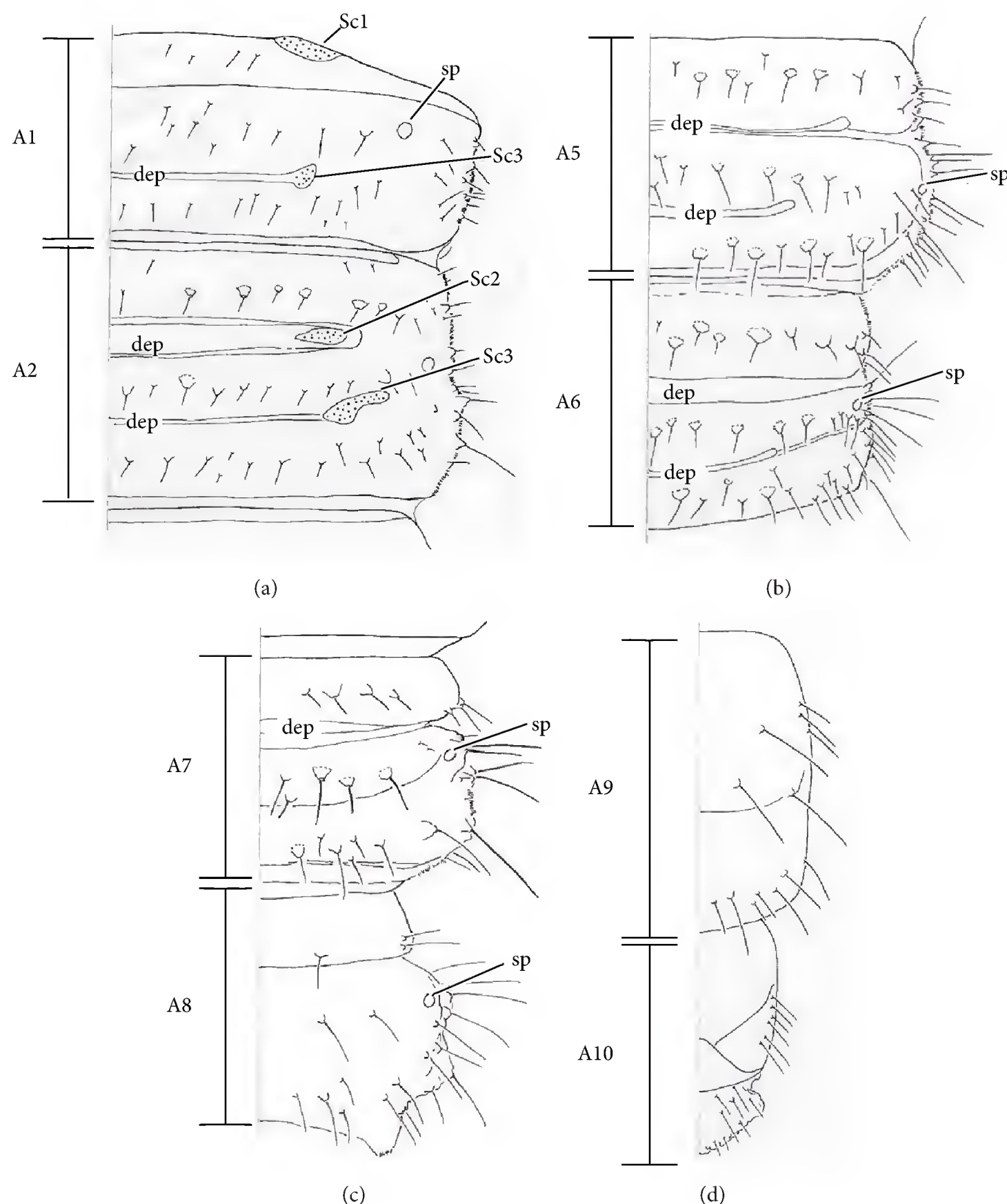


FIGURE 13: Abdomen (dorsal) of *Nothochrysa californica* Banks, second instar. (a) First and second segments. (b) Fifth and sixth segments. (c) Seventh and eighth segments. (d) Ninth and tenth segments. Ax: number of abdominal segment; dep: smooth-surfaced, intrasegmental depression between subsegments; Sc1, Sc2, and Sc3: first, second, and third primary sclerites of each segment; sp: spiracle.

From the above, it is obvious that the choice of an out-group is of crucial importance for the interpretation of an apomorphic versus plesiomorphic state for the various larval characters. Unfortunately, there has been relatively little comparative systematic work that compares hemerobiid and chrysopid larvae (see [22]).

7. Nothochrysine Phylogeny

When Adams [1] revised the subfamily Nothochrysinæ, it comprised seven extant genera and four fossil genera attributed to the Miocene. Later, two extant and eight fossil genera were added, and one extant genus was synonymized [3, 6, 23]. So, now the subfamily includes eight extant genera

and twelve extinct fossil genera from the early Eocene (Ypresian) to the Miocene (Aquitanian, Burdigalian?).

Although Adams [1] included *Nothochrysa* within the subfamily because it retained many archaic and/or unusual features that are not expressed in the Chrysopinae, he pointed out that it has other advanced features that are more typical of Chrysopinae and Apochrysinæ than Nothochrysinæ (e.g., in its development of the pseudomedia and pseudocubitus, its wing folds). And recently, an extensive study of all known fossil chrysopids identified patterns of evolutionary progression in certain venational features among the nothochrysine fossils that support a distinct position for *Nothochrysa* [3]. But, the relationships among the extant genera remain unresolved.

Based on his morphological studies, Brooks [2] proposed that the extant genera of the subfamily fall into two groups.

Nothochrysinæ*Dictyochrysa* – **Naked***fulva* Esben-Petersen

New 1981 [14] [Desc-L1; Draw-L1; Note]

Hypochrysa – **Naked***elegans* (Burmeister)Brauer 1867 [15], as *Hypochrysa nobilis* Heyd. [Desc-L3; Draw-L3; Note]Lacroix 1925, as *Hypochrysa nobilis* Schneider [Note]Principi 1956b, as *H. nobilis* Schneider [Note: svelt, agile, green larva, no mention of debris]

Gepp 1983 [Photo-L3; Note]

Gepp 1988 [Photo-L3; Note]

Díaz-Aranda and Monserrat 1995 [18] [Draw-L1, L3; Note]

Gepp 1999 [Photo-L3]

Díaz-Aranda et al. 2001 [Draw-L1, L3; Note]

Monserrat and Díaz-Aranda 2012 [13] [Desc-L1, L3; Draw-L1, L3; Note]

africana (Kimmins)

Duelli et al. 2010 [4] [Photo-L3]

Tauber 2014 [6] [Desc-L2; Draw-L2; Photo-L2; Note: Synonymy of *Kimochrysa* with *Hypochrysa*, largely based on larval characters]

Tauber and Faulkner, this paper [Draw-L2; Note]

Nothochrysa – **Varied, with both Debris-carrier & Occasional Debris-carrier***californica* Banks

Toschi 1965 [16] [Desc-L1–L3; Draw-L1–L3; Note; Mat: not specified; Beh]

Tauber and Tauber, unpublished data [Mat: bracts from *Quercus* catkins, frass, plant fragments]

Tauber and Faulkner, this paper [Redesc-L2; Draw-L2; Photo-L2]

capitata (Fabricius)*

Withycombe 1922 [Desc-L1–L3; Draw-L3; Note: a few large pieces; Mat: not specified]

Lacroix 1925 [Note: packet loosely attached, not elaborate; Mat: not specified]

Killington 1931 [Note: a few large pieces; Mat: not specified]

Killington 1937 [17], as *Nathanica* [Desc-L1–L3; Draw-L3; Note: information from Withycombe 1922]Kimmins 1939, as *Nathanica* [Desc-L1; Draw-L1]Fraser 1959, as *Nathanica* [Draw-L2 or L3; Note; Mat: not specified]New 1969, as *Nathanica* [Note: L2, L3 – most carried some to much debris; Mat: mainly animal remains; Beh]

Gepp 1983 [Photo-L3; Note; Mat: frass, twisted plant material, lichens]

Barnard et al. 1986 [Desc: dorsal coloration]

Gepp 1988 [Note: packet loose, on central part of abdomen; Mat: bud scales, frass]

Monserrat and Díaz-Aranda 2012 [13] [Desc-L3; Draw-L3; Note; Mat: few large, diverse fragments]

fulviceps (Stephens)

Lacroix 1925 [Note: packet loosely attached, not elaborate; Mat: not specified]

Killington 1935, as *Nathanica* [Desc-L1; Draw-L1; Note; Mat: not specified]Killington 1937 [17], as *Nathanica* [Desc-L1; Draw-L1; Note; Mat: not specified]

Gepp 1983 [Photos-L3, with and without debris; Note; Mat: large pieces of plant material, shed bark etc.]

Gepp 1984a [Photo-L3; Note; Mat: not specified]

Gepp 1988 [Draw-L3, Photo-L3; Note: packet on abdomen; Mat: bud scales, frass, coleophorid cases]

Díaz-Aranda & Monserrat 1995 [18] [Draw-L3; Note; Mat: not specified]

Díaz-Aranda et al. 2001 [Draw-L3; Note; Mat: not specified]

Monserrat & Díaz-Aranda 2012 [13] [Desc-L3; Draw-L3; Note; Mat: few large pieces of different nature]

Pimachrysa – **Naked***fusca* Adams

Tauber & Faulkner, this paper [Desc-L2, L3; Draw-L2; Photo-L2, L3; Note]

Asthenochrysa – **Larvae Unknown***Leptochrysa* – **Larvae Unknown***Pamochrysa* – **Larvae Unknown***Triplochrysa* – **Larvae Unknown**

Box 1: Catalog of larval descriptions for Nothochrysinæ (updated from Tauber et al. [5], where the citations not listed in the references here can be found). * See Monserrat and Díaz-Aranda 2012 [13] regarding problems in distinguishing *N. capitata* and *N. fulviceps*.

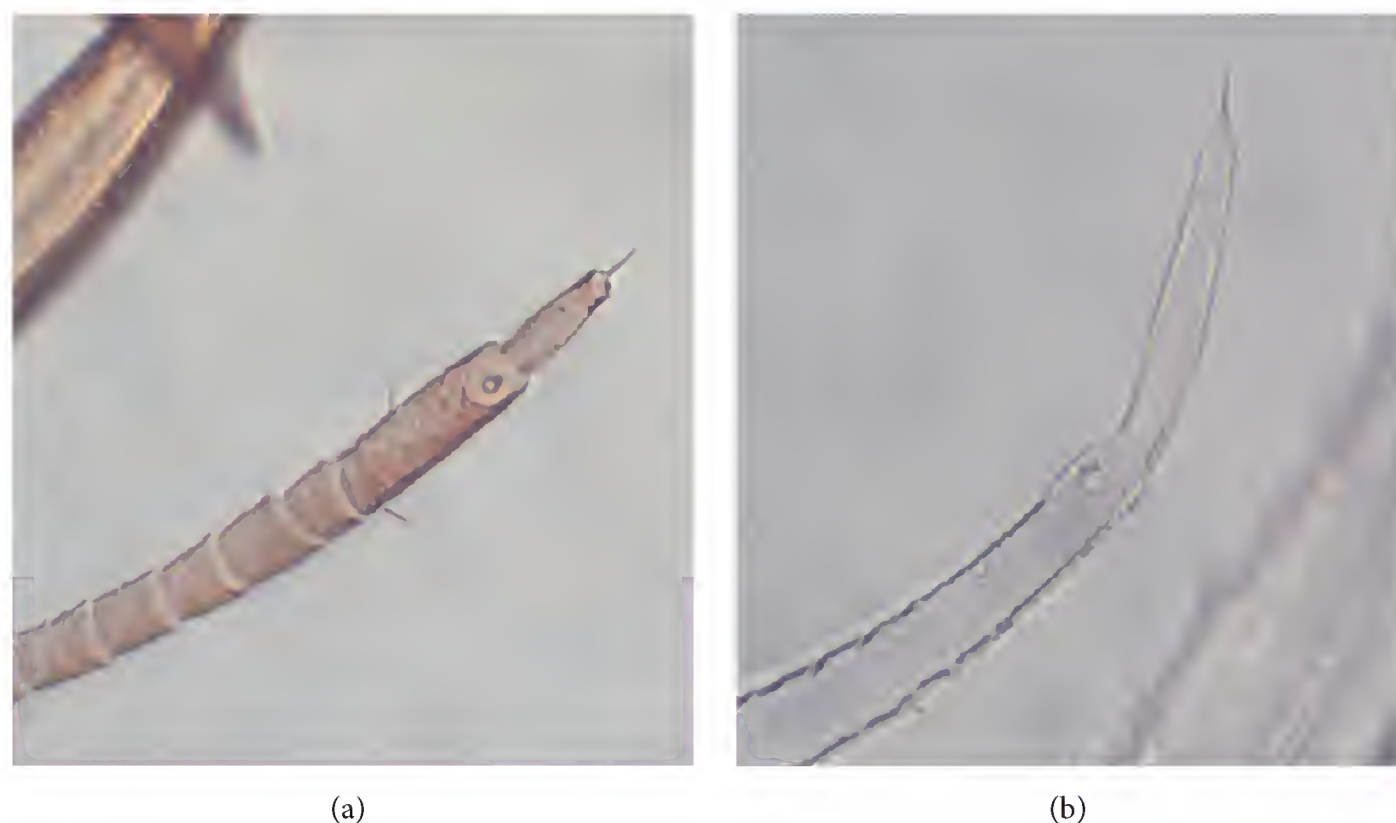


FIGURE 14: Flagellum and distal annulations of pedicel on (a) *Pimachrysa fusca* Adams and (b) *Nothochrysa californica* Banks, second instar antenna. Note: stubby flagellum and short terminal setae on *P. fusca* and elongate flagellum and single, more robust terminal seta on *N. californica*.

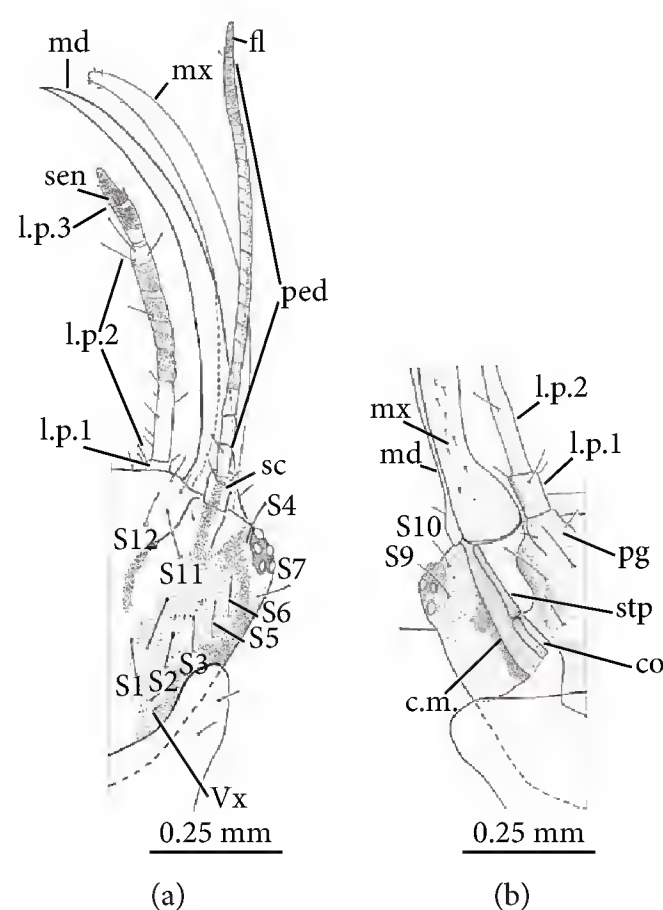


FIGURE 15: Head of *Hypochrysa africana* Kimmins, second instar. (a) Dorsal. (b) co: cardo; c.m.: cranial margin; fl: flagellum; l.p.x: labial palps, number of segment; md: mandible; mx: maxilla; ped: pedicel; pg: palpiger; sc: scape; sen: sensilla; stp: stipes; Sx: primary seta number; Vx: Vx setae.

In his scheme *Nothochrysa* was included with *Dictochrysa* and *Hypochrysa*, separate from *Pimachrysa* and *Kimochrysa*. These groupings subsequently were found to be weak; indeed *Kimochrysa* was synonymized with *Hypochrysa* [6]. Finally, in another study that used molecular and other data, Duelli et al. [4] proposed that the small bodied, pollen-feeding nothochrysine genera (e.g., *Hypochrysa* sen. lat. (including *Kimochrysa*), *Pamochrysa*, *Pimachrysa*, and *Asthenochrysa*) are very similar to each other. These authors concurred with the opinion that although *Nothochrysa* shows some

affinities with these nothochrysine genera, they also resemble Apochrysinae and Chrysopinae in other respects. Given their findings, they conjectured that the small bodied, pollen-feeding nothochrysines represent the most ancient group of extant chrysopids. Moreover, they concluded that pollen-feeding is the likely ancestral condition for chrysopid adults.

It appears from the descriptions of now four of the eight nothochrysine genera (Box 1) that larval characters support the conclusions of Adams, Archibald and Makarkin, and Duelli et al. [1, 3, 4]. The pattern of larval differences between

Pimachrysa (L2, L3)

Larvae naked, without a layer of camouflaging material.

Small to medium sized larvae (L2: ~5.5 mm; L3: 8.5–9.0 mm); shape fusiform, narrow, abdomen rounded, slender.

Cephalic markings obvious, lateral epicranial markings elongate; mesal epicranial and/or postfrontal markings reduced; with a conspicuous median stripe.

Cephalic setae: all primary setae except S10 and S12 present; Vx obscure, probably two setae and a pore.

Antennae with terminal segment (flagellum) stubby, short (0.07–0.08x length of pedicel).

Mouthparts elongate (mandible ~1.4–1.6x longer than head capsule).

Mandible with single seta on basolateral margin.

Thorax without lateral tubercles.

Paired prothoracic sclerites (Sc1) elongate.

Metanotum with three distinct folds, each bearing a transverse band of unhooked setae with acute tips.

Legs fairly long in relation to body; tarsi dark brown throughout.

Abdominal segments II–VII without lateral tubercles, with some lateral setae slightly elongated, but without distinct lateral setae (LS).

Abdominal segments I–VII dorsally with transverse bands of numerous short, acute setae (SMS).

Abdominal segments V–VII without laterodorsal tubercles, without LDS (but with some laterodorsal setae slightly elongated).

Nothochrysa (all instars, unless noted otherwise)

Larvae usually bearing a camouflaging layer of debris.

Large larvae (L3: 9–12 mm; L2: 6–7 mm; L1: 4–5 mm); shape very slightly fusiform, abdomen convex, almost flattened to globular.

Cephalic markings obvious, epicranial markings large, fused with postfrontal markings, occupying almost the entire cephalic dorsal surface.

Cephalic setae: all primary setae including S12 present; Vx = two short anterior setae, pore, single posterior seta, in longitudinally elongate row.

Antennae with terminal segment (flagellum) of variable length.

Pedicel length/flagellar length (= number of times flagellum shorter than pedicel):

N. capitata L3 = ~8.8 (from Figures 47–6 [13])

N. fulviceps L3 = ~13.0 (from Figures 47–12 [13])

N. californica L1 = ~3.6, L2 = 5.1–6.2, L3 = 9.4–9.5

Flagellar terminus and terminal bristle variable.

N. capitata, *N. fulviceps* L3 with rounded flagellar terminus, very short terminal bristle (from Figure 1b [18], Figures 47–6, 47–12 [13])

N. californica L3–L1 with flagellum tapered, slightly elongate seta (setal length/flagellar length: L3 = 0.18, L2 = 0.18, L1 = 0.25)

Mouthparts noticeably longer than the head capsule.

N. capitata, *N. fulviceps* L3 (from Figures 46–1, 46–5 [13])

N. californica L1–L3 (mandible length/head length: L1 = 1.36, L2 = ~1.60–1.65, L3 = 1.30–1.48)

Mandible with two setae on basolateral margin.

Thorax with lateral tubercles present, carrying 1–2 or few elongate lateral setae (LS).

Paired prothoracic sclerites (Sc1) triangular.

Metanotum with transverse row of filiform (hooked or straight) setae posteriorly.

Legs very long in relation to body size; tarsi pale to dark brown.

Abdominal segments II–VII with lateral tubercles well developed, hemispherical, bearing filiform or hooked (“uncinate”) setae (LS).

Abdominal segments VI–VII (sometimes also V, VIII) with laterodorsal tubercles present, bearing single long seta (LDS?) (sometimes also a shorter seta).

Dorsum of abdominal segments I–VI with transverse rows or bands of hooked submedian setae (SMS).

Hypochrysa (L1, L3)

Larvae naked, without a layer of camouflaging material.

Small to medium sized larvae (L2: 6–7 mm); shape fusiform, narrow, abdomen rounded, slender.

Cephalic markings light or dark; lateral epicranial markings narrow, elongate; mesal epicranial and/or postfrontal markings faint, either with a conspicuous median stripe or with pair of small frontal markings.

Cephalic setae: all primary setae including S12 present; Vx obscure, probably two setae and a pore or three setae.

Antennae with terminal segment (flagellum) stubby, short (~0.05–0.06x length of pedicel).

Mouthparts elongate (mandible ~ 1.7 – $1.8\times$ longer than head capsule).
Mandible with single seta on basolateral margin.
Labial palp with terminal segment bearing 4 lateral sensilla.
Thorax without lateral tubercles.
Paired prothoracic sclerites (Sc1) elongate.
Metanotum with three distinct folds, each bearing a transverse band or row of unhooked, blunt to clavate (bacilliform) setae.
Legs of normal size in relation to body; tarsi dark brown throughout.
Abdominal segments II–VII without lateral tubercles, without distinct lateral setae (LS).
Abdominal segments I–VII dorsally with transverse rows or bands of numerous short, blunt to clavate (bacilliform) setae (SMS).
Abdominal segments V–VII without laterodorsal tubercles, without LDS.

Dictyochrysa (L1)

Larvae naked, without a layer of camouflaging material.
Small to medium sized larvae (L1: ~ 2 mm); shape fusiform, narrow, abdomen rounded, slender.
Cephalic markings unknown.
Cephalic setae: S12 absent; S8–10 unknown; Vx short.
Antennae with terminal segment (flagellum) rounded apically, pedicel length/flagellar length = 9 (number of times flagellum shorter than pedicel) (from Figure 3 [14])
Mouthparts nearly twice as long as head capsule.
Number of setae on mandible unknown.
Thorax without lateral tubercles.
Paired prothoracic sclerites (Sc1) triangular.
Metanotum with three distinct folds, each bearing a transverse band of unhooked setae with acute tips.
Legs fairly long in relation to body; tarsi dark brown throughout.
Abdominal segments II–VII without lateral tubercles.
Abdominal segments I–VII dorsally with transverse bands of blunt setae (SMS).
Abdominal segments V–VII without laterodorsal tubercles, without LDS.

Box 2: Summary of larval features of nothochrysin genera with described larvae. In addition to information in this paper; see [6, 13–18].

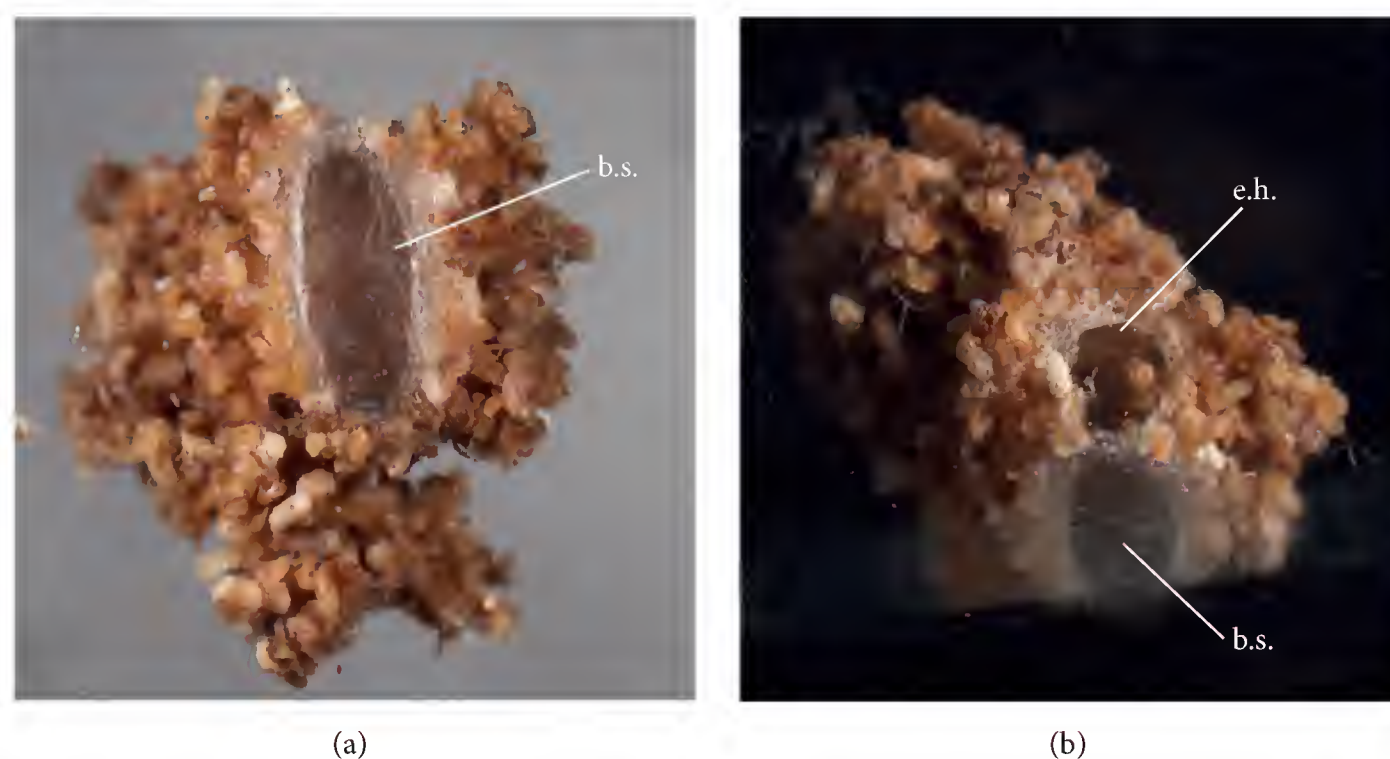


FIGURE 16: *Pimachrysa fusca* Adams, cocoon (Joshua Tree National Park, Riverside Co., CA). (a) Basal surface attached to glass vial. (b) Round exit hole on upper surface (head end) of cocoon. b.s.: basal surface; e.h.: exit hole.

Nothochrysa and the other known nothochrysin genera is consistent with the pattern of variation expressed in adult morphology. Most notable among the larval traits is the difference in larval life style between the small-bodied genera and *Nothochrysa*. Three of the small-bodied genera—*Hypochrysa*, *Pimachrysa*, and *Dictyochrysa*—are now known

to have naked larvae; they have narrow, fusiform bodies that lack lateral tubercles on the thorax and abdomen and are without laterodorsal tubercles on all abdominal segments. In marked contrast, the larvae of the European *Nothochrysa* species and the North American *N. californica* are debris-carriers; they have broad, somewhat flattened bodies, small

but distinct, sclerotized, lateral tubercles on the thorax and abdominal segments A2 through A7, as well as other morphological adaptations for holding debris (Box 1).

It is noteworthy that within the family Chrysopidae, life style (larval nakedness versus debris-carrying) is associated with numerous morphological and behavioral adaptations, and yet it is an evolutionarily very labile trait. Debris carrying occurred very early in chrysopid evolutionary history [24], and both naked and debris-carrying forms have evolved numerous times within the family [5, 24]. Thus, it could be argued that the characters above are redundant—all an expression of the naked versus debris-carrying life styles. However, it is important that, in addition to features associated with life style, the larvae of *Nothochrysa* species also differ from other nothochrysinines in features that appear unrelated to their debris-carrying habit (Box 2). For example, as discussed above, larvae of *Nothochrysa* species show a broad range of interspecific and developmental variation in flagellar length and in the length and structure of the terminal seta(e) on the flagellum. They also differ in their head markings and in the shape and size of the pronotal sclerites. Each of these characters provides independent support that *Nothochrysa* is distinct from the smaller-bodied nothochrysinine genera and that species within *Nothochrysa* present a series of intermediate conditions between the more archaic extant nothochrysinines (e.g., *Dictyochrysa*, *Hypochrysa*, and *Pimachrysa*) and the Apochrysininae-Chrysopinae lineage(s). If this hypothesis proves true, not only would pollen-feeding likely be an ancestral adult feature of the extant Chrysopidae (as proposed by Duelli et al. [4]), but also nakedness would likely be their ancestral larval life style.

(Note: a previous description [6] of the *H. africana* second instar had some errors in the designation of setal numbers on the dorsum of the head. For accuracy in comparisons with *Pimachrysa* and other nothochrysinine larvae, Figure 15 here corrects those errors.)

Disclosure

This study is part of Regional Project W-3185.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

***Neochrysocharis okazakii* (Hymenoptera: Eulophidae) as a Major Parasitoid Wasp of Stone Leek Leaf Miner *Liriomyza chinensis* (Diptera: Agromyzidae) in Central Vietnam**

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Liriomyza chinensis is a major pest of Welsh onion *Allium fistulosum* in Asia but little is known about the abundance of its natural enemies. A field survey was made to explore the major parasitoids of *L. chinensis* leaf miner in central Vietnam. An eulophid parasitoid, *Neochrysocharis okazakii*, comprised more than 95% of parasitoids reared from leaf miner larvae collected in the onion field and 98.3% of leaf miner parasitoids found during searches of onion plants. The mean number of female *N. okazakii* on plants was greater in onion fields with a higher density of *L. chinensis*, and, during searches, a greater proportion of *N. okazakii* was found on onion with more *L. chinensis* mines, suggesting density-dependent parasitoid aggregation. Melanized dead larvae of *L. chinensis* were more frequently found in onion fields with more parasitoids, demonstrating that melanized leaf miners are a good indicator of parasitoid activity. Mean instant rate of host encounter by *N. okazakii* in the field was estimated at 0.077, and the likelihood of a parasitoid finding a host increased with host density. Taken together, these results show that *N. okazakii* is the major parasitoid of *L. chinensis*. Usefulness of this parasitoid in stone leek leaf miner management is discussed.

1. Introduction

Welsh onion *Allium fistulosum* (Liliaceae or Alliaceae) (= green bunching onion or Japanese bunching onion) is a vegetable crop widely cultivated in the world [1, 2]. It is an important part of the fresh market in Asia, particularly in Vietnam, China, and Japan [3, 4]. Welsh onion is grown throughout the majority of region in those countries. Welsh onion is typically grown as an annual though it is a perennial crop.

Pest management is crucial to stable production of marketable Welsh onions. Chemical control, that is, the use of synthetic pesticides, is currently the sole measure (except “hand-picking”) for combating pests of Welsh onion in Vietnam [5]. The situation is similar in other Asian countries including Japan. However, relying too much on chemicals often causes the development of pesticide-resistance and the

resurgence of pest populations [6–8]. This holds true for Welsh onion production in Vietnam [5].

Stone leek leaf miner *Liriomyza chinensis* (Diptera: Agromyzidae) is a common and serious pest of Welsh onion in East and Southeast Asia including Vietnam [9–12]. It often causes unacceptable damage to the marketable parts of green onion. Previous surveys in central and southern Vietnam have shown that almost 100 percent of all leaf miners attacking Welsh onion are *L. chinensis* and that heavy infestation by *L. chinensis* is detected in many Welsh onion fields where insecticides have frequently been applied [6]. Thus, insecticide-based control does not satisfactorily work.

In general, leaf mining insects host many species of parasitoid wasps, and parasitoid wasps can effectively reduce pest leaf miner populations if chemicals that are harmful to them are not used [13–17]. Biological control by preventative or curative releases of commercialized parasitoids may be ideal

in *L. chinensis* management. This approach, however, is not acceptable for Vietnamese farmers because such parasitoids are too expensive for them. Instead, conservation biological control is known as a useful, low-cost tactic [8, 18, 19]. One of the mainstays of IPM of stone leek leaf miner in Vietnam could be conservation biological control.

Very little is known about the impact of parasitoids on *L. chinensis* populations. To date, only a few studies have investigated the parasitoid species complex of *L. chinensis* [11, 20, 21]. The present study was undertaken to determine the major parasitoid species of onion leaf miner in central Vietnam. Field studies were conducted to examine (1) the parasitoid species complex and (2) the relationship between pest infestation levels and parasitoid abundance and activity in the field. The importance of parasitoids and conservation biological control in stone leek leaf miner management in Vietnam is discussed.

2. Materials and Methods

Field studies were conducted from February to March 2005. Study sites are located at Hue and Huong An, lowland areas in central Vietnam. In these sites, Welsh onion is a year-round vegetable, and different growing stages were concurrently present. Welsh onion fields selected for study were conventional farmer's fields with onion plants of vegetative and scape elongation stages; farmers exclusively used Cartap as the chemical control tool to manage insect pests, and no herbicides were applied. A variety of vegetable and ornamental crops such as corn, lettuce, green mustard, soybean, snap bean, sweet potato, and chrysanthemum were grown around study fields.

Leaf miner infestation and parasitoid activity levels were examined in 15 farmer's fields. Inspection of the entire plant was made to search leaf mines. Each field was sampled at four sites. Up to a total of 50 plants per field with 10–15 plants per site were randomly sampled. The numbers of leaf mines and melanized dead leaf miner larvae were counted for each plant. The mean numbers of leaf mines and melanized larvae per plant were used as indices of pest and parasitoid activity at each field.

Also, adult parasitoids on Welsh onion leaves were searched. Preliminary observations had shown that fewer adult parasitoids of leaf miners were observed in the afternoon, possibly because unstable weather conditions were commonly encountered at that time. Because parasitoid activity might be influenced by weather condition and time, field observations were made between 9:00 and 11:00 o'clock on fine days. The number of parasitoids was counted by inspecting the entire onion plant. Female parasitoids were collected for identification. Each field was examined on at least three times (= days). In each field, four sites were chosen, and up to a total of 50 plants per field with 10–15 plants per site were randomly examined.

In addition, direct observations of parasitoid behavior were made to determine if leaf miner parasitoids observed in study fields were associated with *L. chinensis*. Thirteen fields were used in this observation study. When a female parasitoid was found, its behavior was observed and classified as

attacking, searching (antennal drumming), resting, walking, and hopping.

In order to examine parasitoid species that emerged from host larvae, the hosts were collected from Welsh onion fields and reared at the laboratory of Hue University. Collections were made at Hue from March to July 2005. Incubators were not available so humidity and room temperature could not be controlled. Consequently, the quality of onion leaves was not optimal for leaf miner development. Also, stone leek leaf miner is very different from other *Liriomyza* species in its feeding habit. Mines of the former are rather straight, and the visible parts of mines are short and interrupted because larvae alternately mine the outer and inner surface of onion leaves. Mature larvae of *L. chinensis* are often found within onion leaves. For these reasons, we were unable to determine the exact percentage of parasitism. Instead, the number of parasitoid wasps that emerged and the species composition were determined to identify the main parasitoid(s) of *L. chinensis*.

Data were analyzed with the aid of JMP version 9.0 [22]. Regression analysis was done to examine relationships between the two variables of interest.

3. Results

Infestation by *Liriomyza chinensis* was observed in all study fields. Infestation level (i.e., mean number of leaf mines per plant) was variable among fields, ranging from 0.5 to 38.7. The wide range of infestation levels allowed analysis of the relationship between infestation levels and parasitoid presence. In all, 132 adult hymenopteran parasitoids were detected in the field. Among them, 2 species of Ichneumonidae, three species of Braconidae, and one species of Chrysididae were not associated with dipteran leaf miners. Based on the morphology, the remaining 121 adult parasitoids were associated with leaf miners. Among the leaf miner parasitoids, 119 individuals were *Neochrysocharis okazakii* Kamijo (Hymenoptera: Eulophidae), which was found mostly frequently on onion leaves. This parasitoid was detected in 11 out of 15 onion fields.

The mean number of *N. okazakii* was significantly higher in Welsh onion fields with higher leaf mine densities per plant ($N = 15$, $r^2 = 0.52$, $F = 13.84$, $df = 1$, and $P = 0.0026$) (Figure 1). Next, we examined the relationship between the mean number of melanized dead leaf miners per plant and that of *N. okazakii* in fields. A significantly positive relationship was detected between the two ($N = 15$, $r^2 = 0.45$, $F = 10.47$, $df = 1$, and $P = 0.0065$) (Figure 2(a)). Also, more melanized leaf miners occurred in green onion fields with higher numbers of leaf mines per plant ($N = 15$, $r^2 = 0.57$, $F = 17.54$, $df = 1$, and $P = 0.0011$) (Figure 2(b)). The proportion of melanized leaf miners/leaf mines/plant tended to increase with increasing mean number of *N. okazakii* observed ($N = 15$, $r^2 = 0.23$, $F = 3.79$, $df = 1$, and $P = 0.074$) (Figure 3).

In all, the behavior of 91 female *N. okazakii* was observed in the fields. Female *N. okazakii* were often found to hop from leaf to leaf (16.4%) or rest on the leaf (49.5%). They

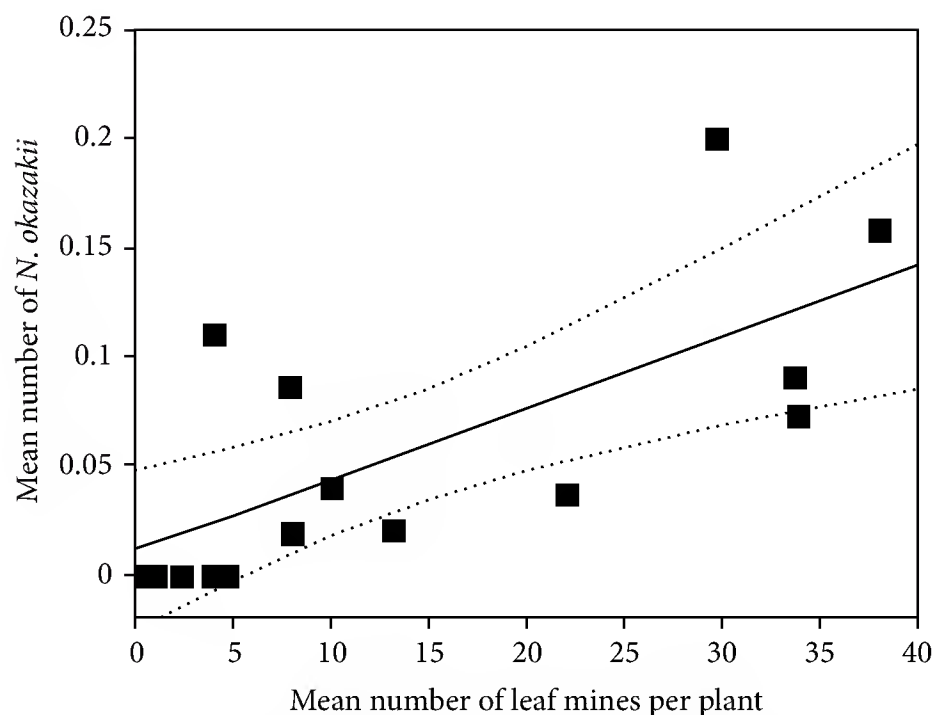


FIGURE 1: Relationship between levels of leaf miner infestation of green onion fields and mean numbers of *Neochrysocharis okazakii* found per plant. Dot lines indicate 95% confidence intervals.

frequently drummed the leaf with their antennae, a behavior associated with searching a host (26.3%). In several cases, it was observed that female parasitoids attacked larvae of *L. chinensis* (7.7%). This value (0.077) can be regarded as an instant rate of host encounter in the field. Because leaf miner species other than *L. chinensis* were not found in study fields, it is likely that *N. okazakii* was associated exclusively with *L. chinensis* in Welsh onion fields.

The proportion of female parasitoids searching for hosts in a given field increased with infestation level in that field ($N = 13$, $r^2 = 0.42$, $F = 6.38$, $df = 1$, and $P = 0.031$) (Figure 4). Logistic regression analysis was done to examine the relationship between host finding success of *N. okazakii* and leaf miner density. In this analysis, the relationship between parasitoid behavior (attacking a host or not) on an onion plant and the number of leaf mines on that onion plant was examined. The analysis showed that successful host finding, that is, the likelihood of females attacking a host, tended to increase with leaf miner density per plant ($N = 91$, $r^2 = 0.104$, $\chi^2 = 5.12$, and $P = 0.024$) (Figure 5).

In all, 216 parasitoid individuals emerged in the laboratory from onion leaves containing *L. chinensis*. Only three species of hymenopteran parasitoids were obtained, and the majority of them (96.8%) were *N. okazakii*. The other two species were *N. formosa* (Westwood) and *Diglyphus isaea* (Walker). Thus, *N. okazakii* was the major parasitoid of stone leek leaf miner in study areas.

4. Discussion

In the present study, direct field observations were first made for the presence of leaf miner parasitoids searching for hosts in Welsh onion fields. Additional surveys were done to examine the parasitoid complex of stone leek leaf miner by collecting onion leaves containing the leaf miner larvae. Both field and laboratory surveys together demonstrated that

more than 95% of parasitoids detected were *Neochrysocharis okazakii*. Our results show that *N. okazakii* is the most important parasitoid species attacking *Liriomyza chinensis* in lowland areas of central Vietnam. *Neochrysocharis okazakii* is a solitary endoparasitoid (Ueno, personal observations). Although information on the life history of *N. okazakii* is limited, available literature suggests that *N. okazakii* is polyphagous like other eulophid parasitoids attacking *Liriomyza* spp. [23, 24]. *Neochrysocharis okazakii* has been found emerging from other leaf miner species, such as *Liriomyza trifolii* and *L. sativae* in Japan, although *N. okazakii* is not considered a major parasitoid of such leaf miner species [12, 17, 23–26].

In the present field observations, parasitoids found attacking stone leek leaf miner were exclusively *N. okazakii*. During the same period as our study, parasitoid abundance was also examined in vegetable fields (other than Welsh onion) where insecticides, mostly Cartap, were applied, and at least four parasitoid species including polyphagous eulophids were searching for leaf mines caused by *L. sativae* on tomato, yard-long bean, and pumpkin (Ueno and Tran, personal observations). Hence, *N. okazakii* is not the only leaf miner parasitoid that occurs in lowland areas of central Vietnam during spring months. Nevertheless, in our study, *Liriomyza chinensis* did not host parasitoids other than *N. okazakii*. Similarly, Tran et al. [21] found that *N. okazakii* occupies more than 90% of parasitoids emerging from onion leaves infested by *L. chinensis*. However, on other sprayed vegetable fields such as tomato and bean fields, the relative abundance of *N. okazakii* among parasitoid species complexes is less than 10% [21]. Species diversity of parasitoids may be very poor for stone leek leaf miner in Vietnam. Curiously, species diversity of parasitoids was also poorer on *L. chinensis* than for *L. sativae* and *Chromatomyia horticola* (Goureau) in China [20].

The low diversity of parasitoids in our study may reflect the fact that *L. chinensis*, which is a specialist of *Allium* spp., attracts a few parasitoid species. Alternatively, the characteristic odor of green onion may have a repellent effect on other parasitoids, or the plant may be a poor environment because of its architecture or lack of refuge from direct sunlight. More likely, it was simply due to the fact that insecticides had been applied [27]. In our study, we could not sample unsprayed fields because all fields had been sprayed with Cartap and could not assess the diversity of parasitoids in unsprayed onion fields. Tokumaru [11] recently reported that 6 species of parasitoid wasps are associated with onion leaf miner in unsprayed fields in Japan. This fact may imply that *N. okazakii* population in our study field showed a level of resistance to Cartap whereas the other parasitoids did not. In our fields, other pests like *Spodoptera exigua* (Hübner) and *S. litura* F. were also abundant. This is curious because Cartap is an insecticide against which resistance is slow to develop [28]. It appears that, for unknown reasons, the applied pesticide did not work well in our study area. Although the effect of Cartap cannot completely be excluded, we believe it did not strongly affect the main result presented here. Factors affecting the makeup of parasitoid communities across different crops remain to be determined in future research.

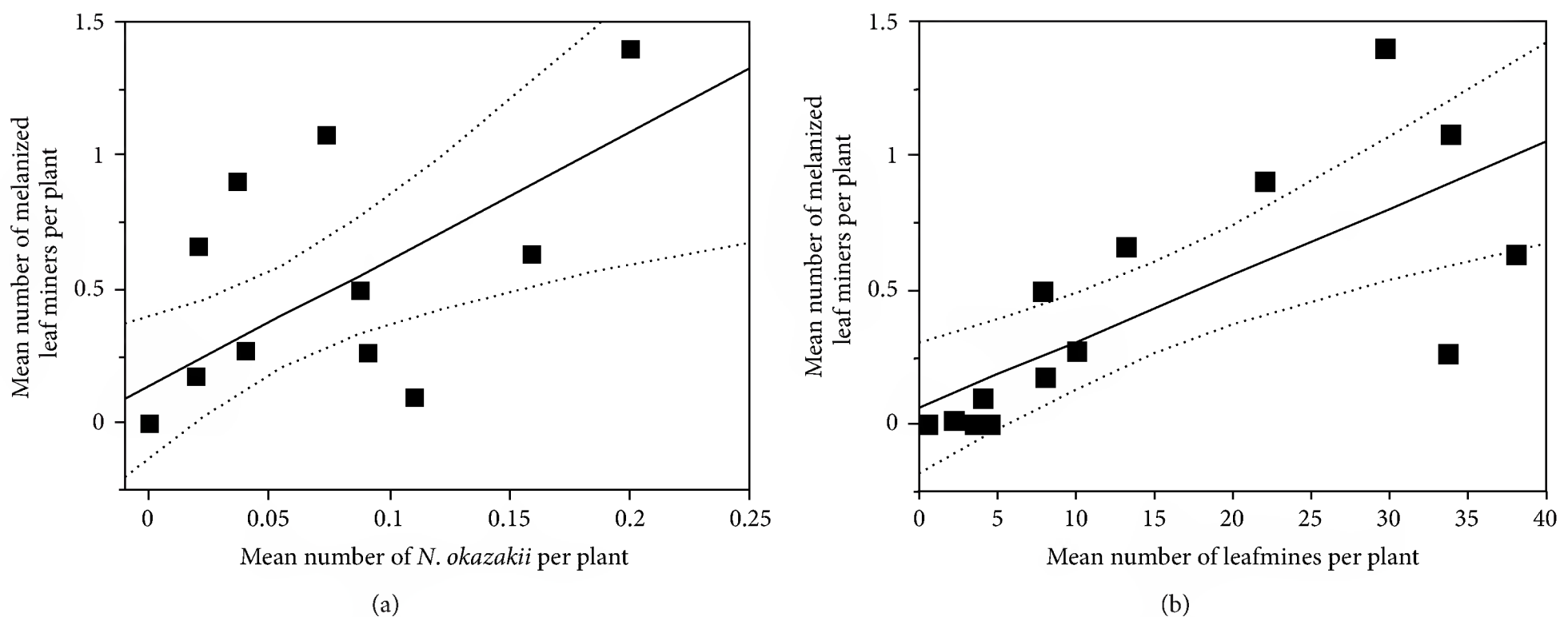


FIGURE 2: Relationships between mean numbers of melanized leaf miner larvae per plant and levels of leaf miner infestation of green onion fields (a) or mean numbers of *Neochrysocharis okazakii* found per plant (b). Dot lines indicate 95% confidence intervals.

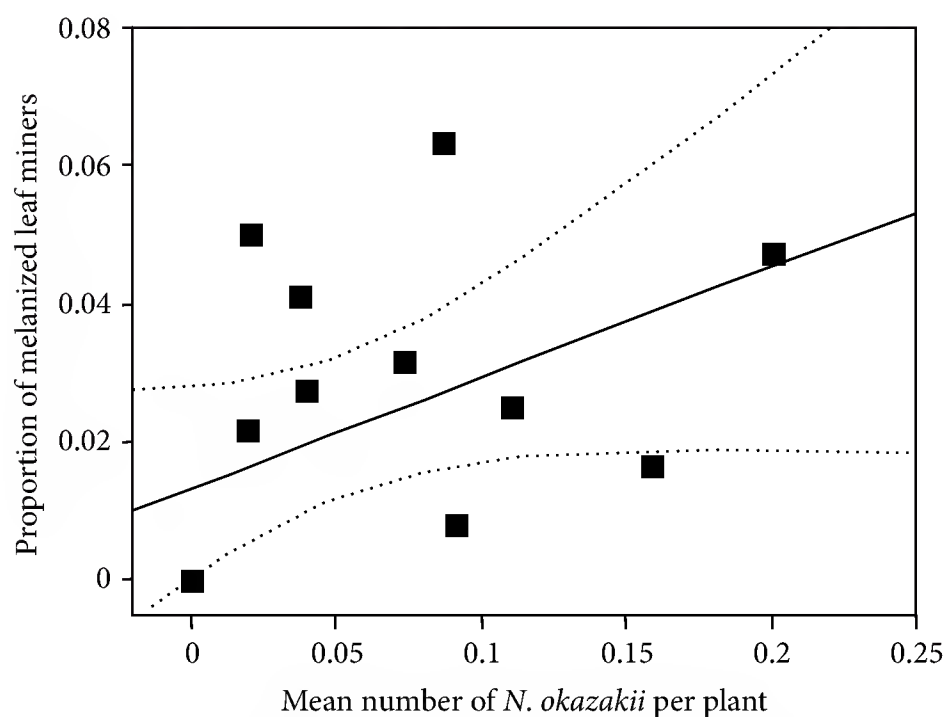


FIGURE 3: Relationship between mean numbers of melanized leaf miner larvae per plant and mean numbers of *Neochrysocharis okazakii* per plant. Dot lines indicate 95% confidence intervals.

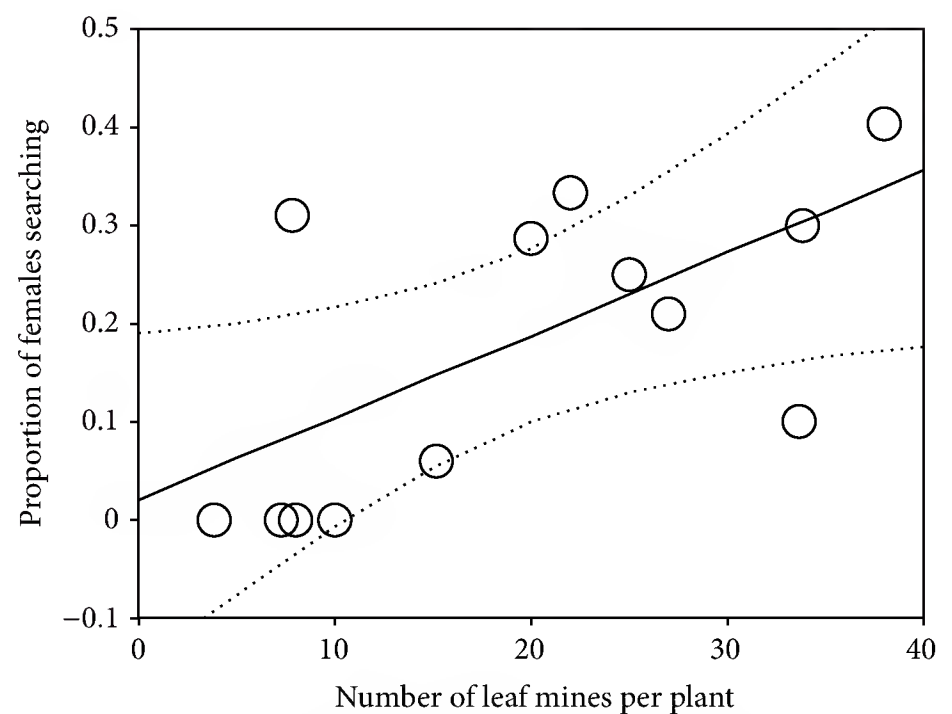


FIGURE 4: Relationship between levels of leaf miner infestation and proportions of female *Neochrysocharis okazakii* searching for hosts. Dot lines indicate 95% confidence intervals.

The present study has shown that melanized dead leaf miner larvae can be a good indicator for the presence and abundance of parasitoids in Welsh onion fields. Melanized leaf miners are visibly prominent, and the presence for dead leaf miners can readily be recognized. Destructive host feeding by female parasitoids probably causes quick mortality of leaf miners, making their body color black due to melanization; *L. chinensis* larvae attacked and oviposited by *N. okazakii* do not normally turn to black after parasitism. The presence of many melanized leaf miners is a clear sign that *N. okazakii* is abundant and can be used when monitoring parasitoid activity in the fields.

Very few studies have evaluated the activity of female parasitoids of leaf miners in the field. The majority of studies assessing parasitoid performance so far have been

based on parasitism rates in the field. In the present study, direct observation was made in the field to examine the relationship between densities of host leaf miner and adult parasitoid. The results showed that more adult females of *N. okazakii* are observed and found searching in onion fields with higher *L. chinensis* densities, suggesting that female *N. okazakii* are attracted to Welsh onion fields with high host densities. A density-dependent response to host of parasitoid may support a successful biological control program [29]. *Neochrysocharis okazakii* may prove to be a good biocontrol agent of the leaf miner in onion fields of Vietnam.

The instant rate of host encounter was estimated at 0.077 under field conditions. Because very few studies have estimated successful host encounter rate by leaf miner parasitoids in the field, it is difficult to judge whether the value is relatively high or low compared with other leaf miner

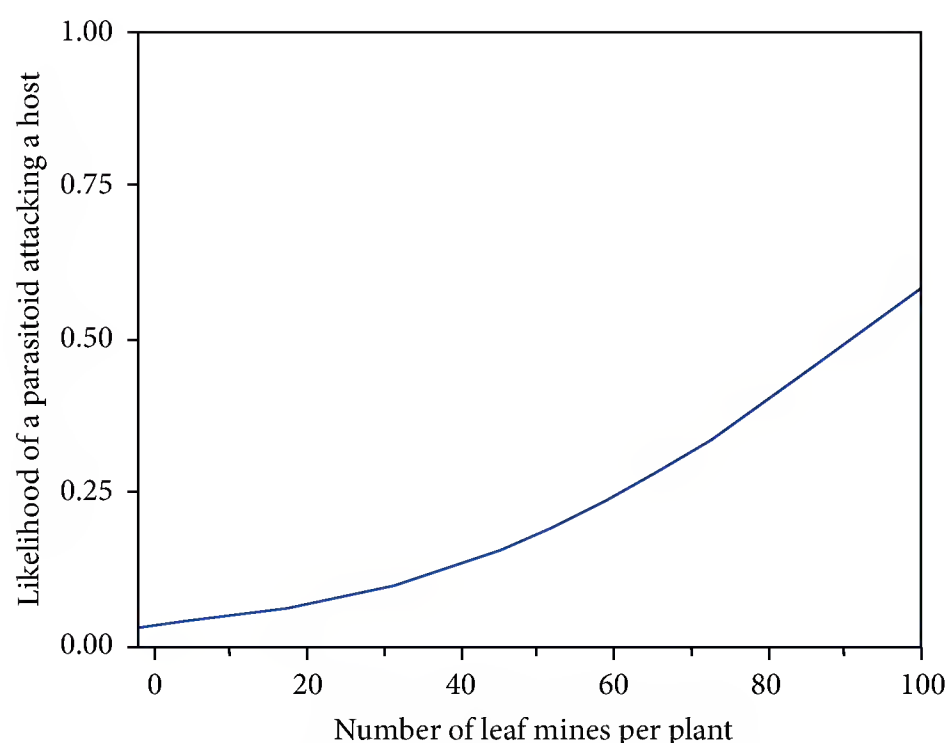


FIGURE 5: Relationship between leaf mine density on an onion plant and likelihood of a female *Neochrysocharis okazakii* successfully finding a host on that plant. A logistic regression curve is illustrated. Data plots are not shown for simplicity.

parasitoids including commercialized species such as *Diglyphus isaea* (Walker), *Neochrysocharis formosa* (Westwood), and *Dacnusa sibirica* Telenga. Nonetheless, our approach in the present study may be useful in assessing the foraging effectiveness of other leaf miner parasitoids.

Pest management strategies should not rely on a single control practice. IPM practices that integrate a variety of tactics should be used [6–8, 30]. The inclusion of different management tactics is required in Welsh onion production in Vietnam. For economic reasons, Vietnamese farmers cannot support high cost pest management practices, and the adoption of close attention should be given to producing the highest possible yield at the lowest cost. In particular, the adoption of low-cost practices that avoid the build-up of insect pests should be emphasized in the country. The conservation of natural enemies is generally simple and cost-effective and may be an important and readily available practice [18, 19]. For successful conservation biological control, farmers' education is necessary together with scientific studies for evaluating the impact of parasitoids on *L. chinensis*.

To date, the importance of natural enemies has not been studied in Welsh onion fields in Vietnam. It is not known whether parasitoids can keep onion leaf miner density below damaging levels if disruptive insecticides are not used. The importance and impact of parasitoids, including *N. okazakii*, should be examined in unsprayed fields. No tests have been conducted to determine which pesticides can be used safely without disrupting *N. okazakii* populations. Also, the possibility that *N. okazakii* demonstrates a level of insecticide resistance is worthy of testing. These tests are essential for the practice of conservation biological control because the use of selective insecticides should not negatively impact the activity of the parasitoid. The usefulness of conservation biological control is worth accessing for Welsh onion production in Vietnam.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Life History of an Endangered Marine Insect *Halovelina septentrionalis* Esaki (Hemiptera: Veliidae)

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Halovelina septentrionalis Esaki is one of the endangered marine water striders found along the Japanese coast. It is of primary importance to investigate its life history in the field so as to develop conservation measures as well as to understand how this species has adapted to marine environments. We studied its life history in Misaki on the southern part of the Miura Peninsular near Tokyo, ca. 35°N, probably the northernmost locality not only for *H. septentrionalis* but also for any *Halovelina* species, most of which are found in warm tropical or subtropical waters. The southern part of the Miura Peninsular has jagged coastlines with deep inner bays. Adults and nymphs were found skating along the rocky shore of inner bays with overhanging vegetation nearby. This species appeared to have at least 2 generations a year and to overwinter in the adult stage. We discussed its overwintering strategy in comparison to those of two other Japanese marine water striders.

1. Introduction

Out of more than one million extant insect species, only a few thousand species are found in the marine environment [1, 2]. Water striders and their allies (Heteroptera: Gerromorpha) are probably the most successful insect group in colonizing in the marine habitats [3]. More than 150 species representing five families of Gerromorpha are marine, occupying the two-dimensional niche, that is, the sea surface, and most of them live in warm tropical or subtropical waters between 30°N and 30°S [3–5].

Along the Japanese coast, 6 species of marine Gerromorpha are known to live. All of them are now designated as endangered or nearly endangered by the national government and/or local authorities due to the population decrease caused by coastal developments and seawater pollution [6]. Two species of endangered marine Gerromorpha, *Halovelina septentrionalis* Esaki and *Halobates japonicus* Esaki, were recorded by Esaki [7–9] along the coast of Misaki on the Miura Peninsular near Tokyo, ca. 35°N, probably the northernmost localities for these two species. For the last several decades the coastlines around this area have been drastically

developed and altered by the growth of big cities such as Tokyo. Surveys conducted in 2011 and 2013 for the two species in the southern part of the Miura Peninsular including Misaki revealed that *H. japonicus* was probably extinct [10]. As for *H. septentrionalis* (Figure 1(a)), they were found in a few inner bays. In most cases, a handful of individuals were found skating on the sea surface close to the shore. Considerably abundant populations were found only at a small number of sites that still to some extent retain the features of natural coasts, such as a cove conserved by citizen groups or some tiny coastal spots hardly accessible from the land due to bad road connections. Little is known about the ecology of *H. septentrionalis* in the natural habitat except for a brief account of its biology and behavior based on the observations by Esaki [7]. It is of primary importance to study its life history in the field so as to develop conservation measures as well as to understand how this species has adapted to marine environments. With the above in mind, we conducted a year-round survey of *H. septentrionalis* in one of the inner bays around Misaki. Most other *Halovelina* species inhabit areas among coral reefs in warm tropical or subtropical waters [11–13]. Therefore, it is quite intriguing how *H. septentrionalis* has

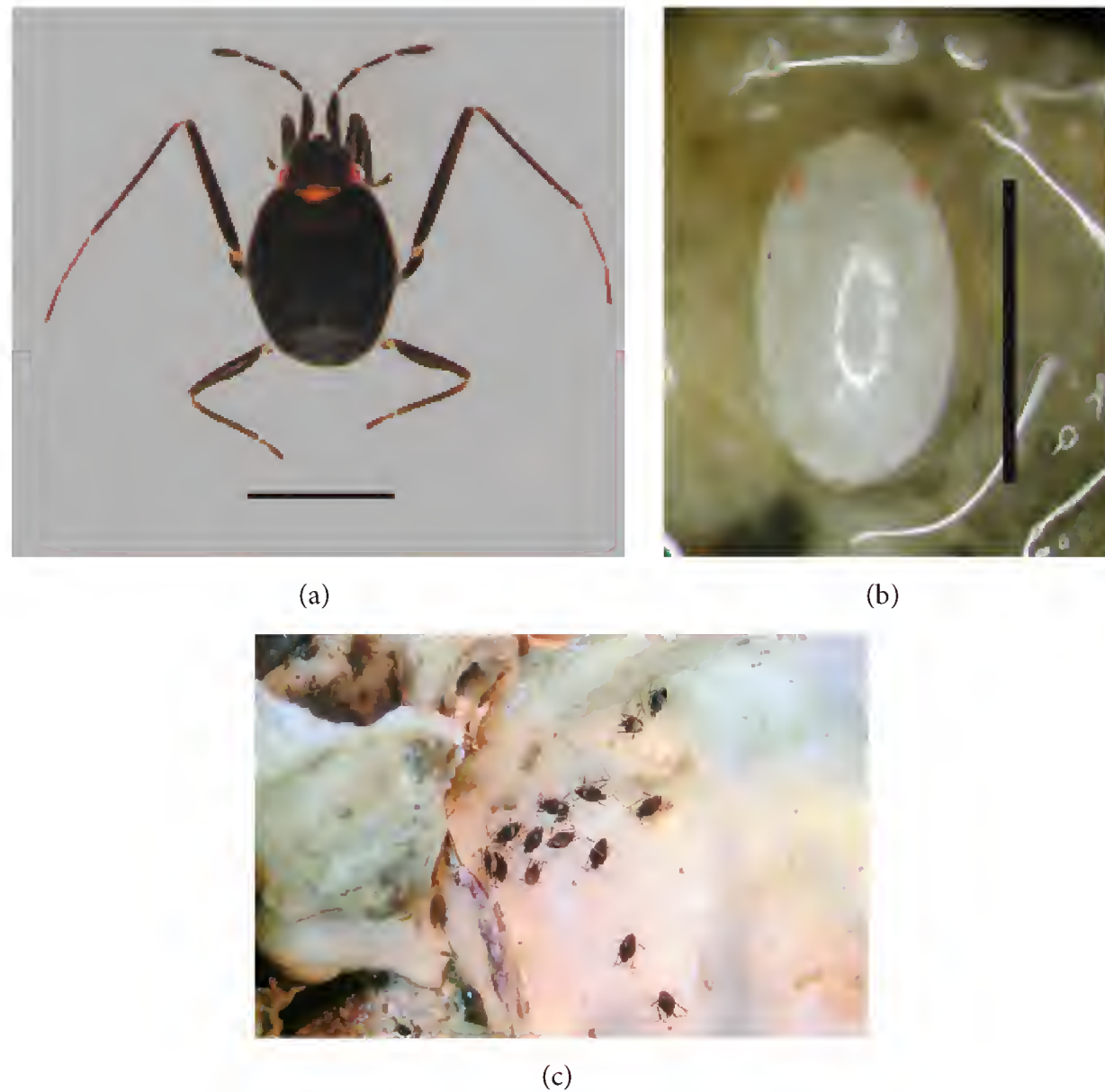


FIGURE 1: *H. septentrionalis*. (a) Male adult. Scale = 1 mm (Photo by T. Ikawa). (b) Developing egg with red eye-spots showing through. Scale = 0.5 mm (Photo by T. Ikawa). (c) Overwintering individuals in an oyster shell (Photo by H. Era).

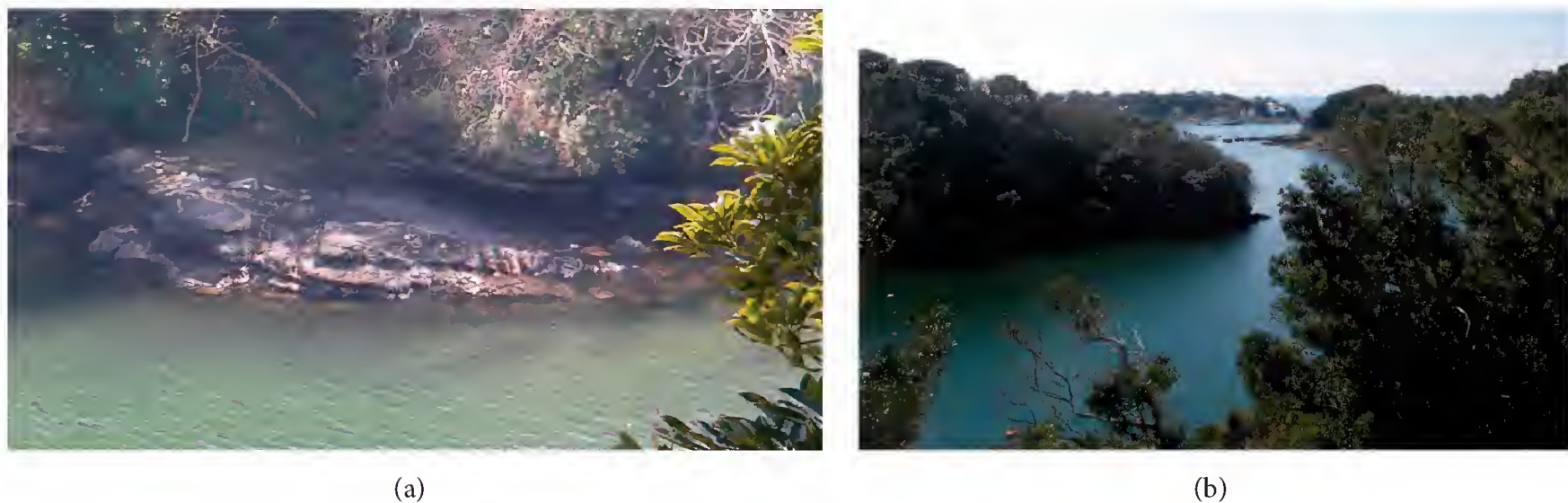


FIGURE 2: Habitat of *H. septentrionalis*. This species is found along rocky shores with overhanging vegetation nearby (a), in a calm inner bay protected from strong winds and waves (b).

survived along the rocky shore of Misaki, in the temperate climate zone of Japan, where snow falls and the sea surface temperature drops below 15°C during winter.

2. Materials and Methods

2.1. Study Site. Our study was conducted along the shore of an inner bay in the Misaki area in the southern part of the Misaki Peninsular, Kanagawa, Japan (35°10'N 139°37'E). The

southern part of the Miura Peninsular has jagged coastlines with deep inner bays as is seen in Figure 2(b). Some other marine water striders recorded in Japan, *Halobates matsumurai* Esaki and *Asclepios shiranui* (Esaki), are also found in such inner bays [14–18]. The study site was characterized by a rocky shore under a cliff with overhanging vegetation nearby (Figure 2(a)), difficult to access from the land. We visited the study site on vessels owned by Misaki Marine Biological Station, School of Science, The University of Tokyo, hereafter referred to as MMBS.

2.2. Surveys and Samplings. Once a month from March 2014 to April 2015 excluding April 2014, we made intensive surveys along the coast in the study site. We searched for eggs, nymphs, and adults of *H. septentrionalis* on the sea surface, on the rock surfaces, in the holes and crevices of rocks, and in empty oyster shells on the rocks. Most adults and nymphs were found skating on the sea surface. In order to examine seasonal changes in the composition of adults and nymphs, from the shore, we collected samples off the sea surface using insect nets with an opening of 25 cm in diameter. The survey dates in 2014 were March 7, May 15-16, June 12-13, July 24-25, August 13, September 10, October 10, November 5-6, and December 8. Survey dates in 2015 were January 7-8, February 6, March 5-6, and April 2-3. The sampling was conducted once during each survey if adults and/or nymphs were found.

All the specimens collected were preserved in 99% ethanol and kept at the University of Morioka for further studies in the laboratory. Under the stereomicroscope adults were sexed and nymphs were sorted into developmental stages by measuring representative leg segments. To examine seasonal changes in the egg load of females, we dissected 20 females from each month, counted mature eggs in the ovarioles, and measured the length and width of the largest mature egg from each female.

3. Results

3.1. Appearance of Adults and Nymphs. As is shown in Figure 3, adults were found from May to November 2014 and in April 2015. Nymphs were found from July to November 2014. During the spring and summer, many mating pairs were observed and during the autumn the number of mating pairs appeared to decrease. The proportion of females was higher than that of males in May, September, October, and November of 2014 and April of 2015. The majority of mating pairs and single adults were skating in sparse aggregations along the shore. Some adults were skating as singles. From July, nymphs appeared. Nymphs were aggregating with adults or skating singly. There were very few young nymphs found during the surveys. Occasionally, a few adults or nymphs were observed jumping on the rock. So far, no individuals were found in the holes and crevices of the rocks.

During the cold season, that is, in the month of March 2014 and during the period from December 2014 to March 2015, neither adults nor nymphs were found in the study site. However, a number of overwintering adults were found in an empty oyster shell on a rock along the shore in a bay near the study site around 11:30 p.m. on December 21, 2014 (H. Era, personal communication). According to Mr. Era, the empty oyster shell was opened with an abalone scraper because it was tightly closed except for a very narrow chasm between valves through which *H. septentrionalis* would barely be able to enter. They were alive but very inactive. Judging from the picture provided by Mr. Era (Figure 1(c)), there were 14 or 15 individuals of which, at least, 11 were female adults, probably 3 were male adults. There was one unidentifiable individual. The oyster shell was attached to the rock at around the lowest sea level which was underwater most of the day. Although several other empty oyster shells were opened and examined

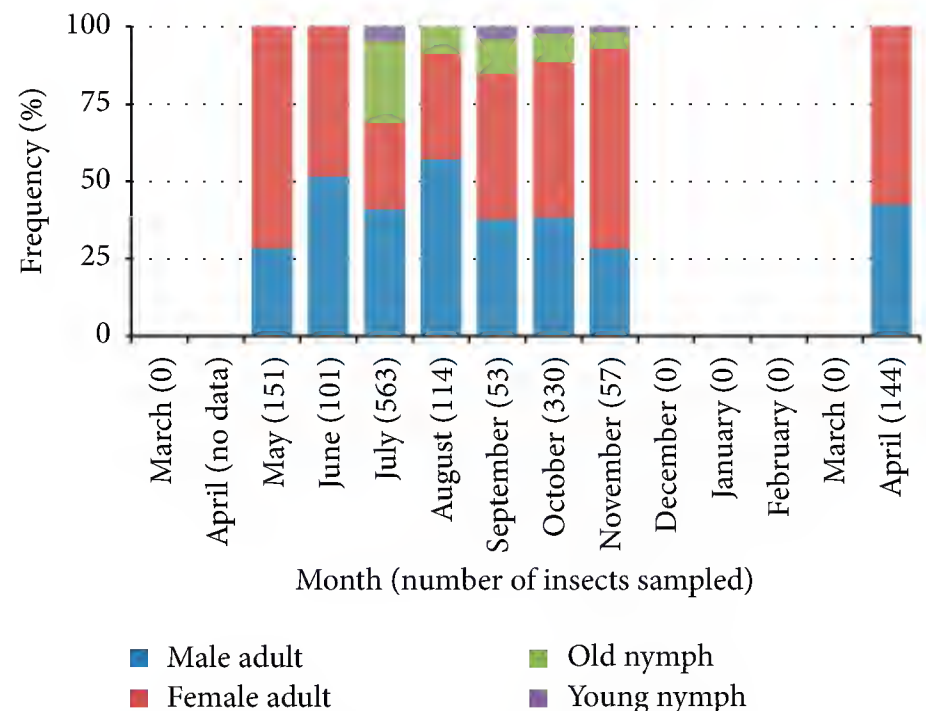


FIGURE 3: Seasonal changes in the composition of developmental stages of *H. septentrionalis* from March 2014 to April 2015.

on the same day, no other *H. septentrionalis* was found. The air temperature was 2°C and the sea surface temperature was 14°C. Since then, we have opened and examined numerous empty oyster shells at the study site but no overwintering individuals have yet been found.

3.2. Egg Load of Adult Females and the Search for Eggs in the Field. The egg loads of females of each month are shown in Figure 4. In late autumn (October and November 2014) and in early spring (April 2015), no females had mature eggs. In May 2014, 50% of females had mature eggs. In June 2014, all the females had mature eggs. From July to September 2014, 65%–100% of females had mature eggs. The number of mature eggs per female was at most 4.

Table 1 shows the average length and width (mm) of the largest mature egg of each female from May 2014 to September 2014. The length ranged from 0.676 mm to 0.703 mm and the width from 0.313 mm to 0.320 mm. Compared to the average size of adult females (body length = ca. 2 mm), the egg size was quite large and the body cavities of females were filled with eggs. The egg shells of *H. septentrionalis* were soft, different from the hard egg shells of other marine water strider *Halobates matsumurai* and *Asclepios shiranui* that overwinter in the egg stage.

In spite of an intensive search during the surveys we found no eggs in the study site. However, females kept in a tank with seawater and rocks in the laboratory laid eggs on a rock around the water surface (Ikawa and Miyata, unpublished observation; Figure 1(b)).

4. Discussion

4.1. Life Cycle of *H. septentrionalis*. Our study showed that (1) no individual was found at the study site during the winter; (2) only adults appeared in spring while nymphs did not appear until summer; (3) both adults and nymphs were observed until late autumn (Figure 3). These results and the fact that a number of overwintering adults were found in the

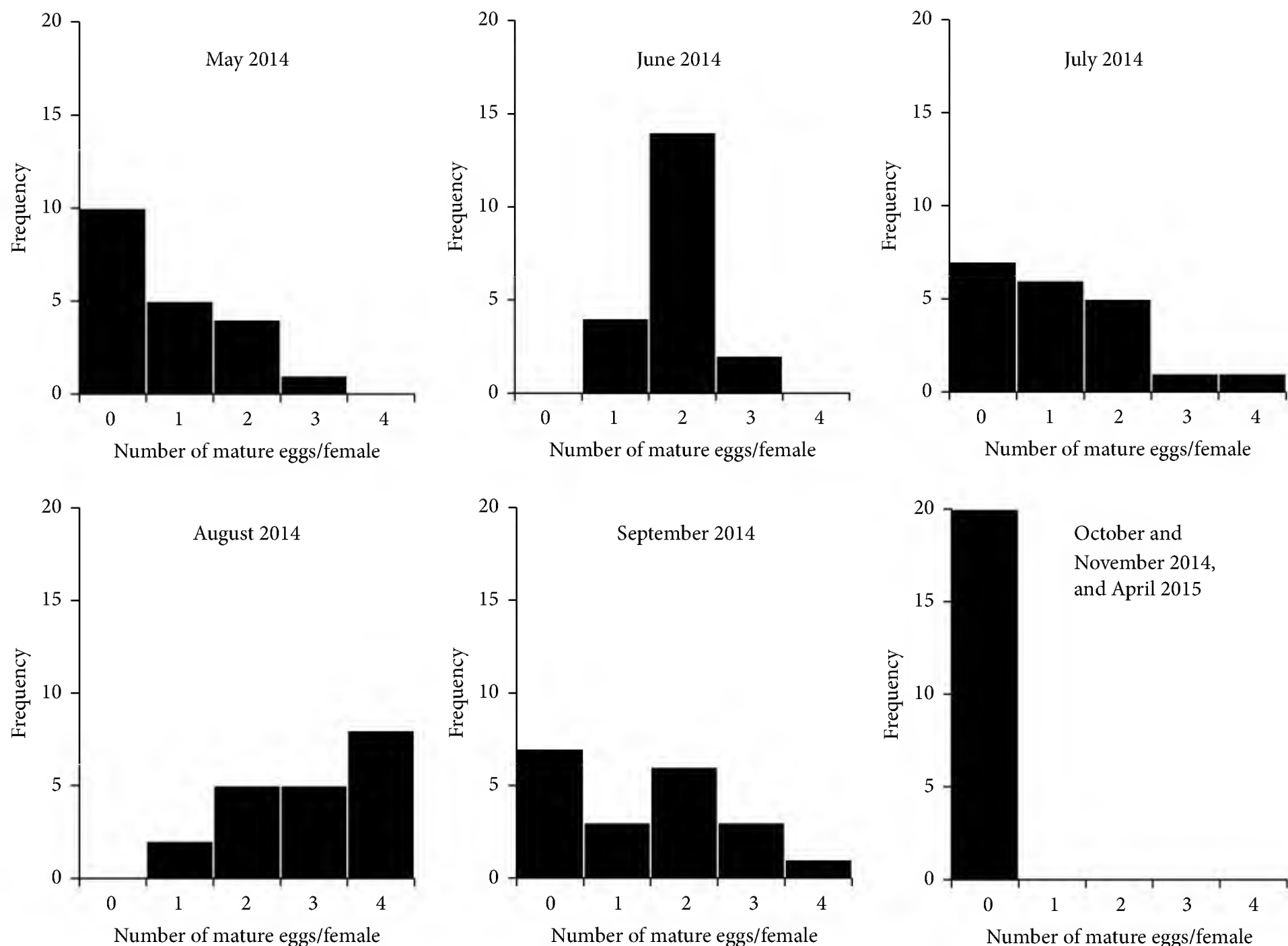


FIGURE 4: Histogram of number of mature eggs/female from May 2014 to April 2015.

TABLE 1: Average length and width (mm) of largest mature egg in a female collected in 2014.

Date of sampling	Number of eggs measured	Length and width (average $\pm$ SD) of mature egg	
		Length (mm)	Width (mm)
May 15	10	0.676 ± 0.0300	0.313 ± 0.0215
June 12	19	0.687 ± 0.0165	0.324 ± 0.0164
July 24	11	0.687 ± 0.0171	0.322 ± 0.0164
August 24	19	0.692 ± 0.0300	0.321 ± 0.0154
September 10	13	0.703 ± 0.0262	0.320 ± 0.0183

empty oyster shell in December (H. Era, personal communication) strongly suggest that *H. septentrionalis* overwinter in the adult stage. After overwintering, adults would have appeared on the sea surface and mated, at latest, by April. As shown in Figure 4, in April no adult females had mature eggs, in May 50% of females had mature eggs, and in June all the females had mature eggs. This suggests that the oviposition of females would have started in late April or early May and lasted for a few months. The nymphs that appeared in July would have been hatched from these eggs and have become adults in August or after. Nymphs observed in late autumn

would have died before becoming adults. Mating pairs were observed until November. However, there were no eggs in females' ovarioles in late autumn. Thus, *H. septentrionalis* probably overwinter in the adult stage and there may be at least 2 generations a year.

4.2. Eggs in the Field. Esaki [7] found no eggs of *H. septentrionalis* during his survey along the coast. We also did not find eggs in the field. However, the fact that females kept in a tank laid eggs on a rock (Ikawa and Miyata, unpublished observation) suggests that the oviposition site of *H. septentrionalis* would be on the surface of the rocks in the field. Kellen [19] found no eggs of *H. bergrothi* Esaki in the coast of Samoa but, in the laboratory, females laid eggs in the holes of rocks in a tank.

4.3. Comparison of Overwintering Strategies among Three Marine Water Striders. *Halobates matsumurai* Esaki live along the southwestern coast of Japan [14, 20]. *H. matsumurai* lays eggs on the sandstones along the shore mostly above the average sea level and overwinters in the egg stage [17]. Overwintering eggs stay on the sandstones for more than 6 months from late autumn to spring. The eggs of *H. matsumurai* are covered with hard shells, presumably serving

to protect them from desiccation, solar radiation, and storms. Laying eggs above the average sea level would serve to keep them from the damage of wave action and thus to survive a long overwintering period. *Asclepios shiranui* that often cooccurs with *H. matsumurai* also has eggs with hard shells and overwinters in the egg stage [17]. This species is suspected of having the same overwintering strategy. On the other hand, for *H. septentrionalis* natural selection has favored overwintering in the adult stage. Overwintering adults were found in an empty oyster shell around the lowest sea level which was underwater most of the day (H. Era, personal communication). It has not been proved yet if this is always the case with overwintering *H. septentrionalis*. Undoubtedly, however, overwintering underwater would be one of the best adaptive strategies to survive the cold season, because the seawater temperature is much warmer than the air temperature. It would be possible for *H. septentrionalis* to survive underwater for a long time through the use of air trapped among the thick hydrofuge hair which covers the body surface, especially because the metabolic rate of overwintering adults would be low thereby requiring little oxygen. Thus, each marine water strider appears to have developed its own adaptive strategy to survive the winter of the temperate climate zone.

5. Conclusion

This paper focuses on the life history of the endangered marine water strider *H. septentrionalis* found in Misaki, Japan, ca. 35°N, the northernmost locality of any extant *Halovelia*-species, most of which live in the tropical/subtropical waters. The results suggest that this species has at least two generations a year and overwinters in the adult stage, presumably staying in some sort of shelter such as an empty oyster shell which remains underwater most of the day. This overwintering strategy differs from those of the two other previously studied Japanese marine water striders.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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